CHRISTIAN PALMBERG

Androgen Sensitivity of Prostate Cancer

Special Reference to Prognostic Factors and Androgen Receptor Gene Amplification

University of Tampere
Tampere 2000

Androgen Sensitivity of Prostate Cancer

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building K,
Medical School of the University of Tampere,
Teiskontie 35, Tampere on December 5th, 2000, at 12 o'clock.

University of Tampere
Tampere 2000

To my father, *Harry Willehard Palmberg*, who died of prostate cancer on the 21th February 1990.

To Laura, Ulrika and Rasmus

ABSTRACT

The purpose of this thesis was to investigate the androgen sensitivity of prostate cancer and to identify prognostic and predictive markers for endocrine-treated prostate cancer.

In study I, 236 endocrine-treated prostate cancer patients were evaluated retrospectively for a decline in PSA during the first year of their treatment. PSA was shown to be an independent factor of prognosis of prostate cancer specific survival [$p<0.001$]. It was also shown that only 6% of primary prostate cancers do not show biochemical response to androgen deprivation therapy.

In study II, the primary androgen-insensitive prostate tumours were characterized immunohistochemically by analysing the apoptotic and the proliferating index and the expression of p53. Patient groups consisted of ten primary androgen-insensitive and twenty androgen-dependent prostate cancers. The apoptotic index was found to be significantly [$p=0.0001$] lower the primary androgen-insensitive group.

In study III, biological and clinical characteristics were retrospectively studied in fifty-four endocrine-treated prostate cancer patients with local recurrence of the disease. Fifteen out of the fifty-four [28%] of the tumours showed an amplification of the androgen receptor (AR) gene detected by fluorescence in-situ hybridization. The presence of the AR gene amplification was associated with both the degree and the duration of response to primary hormonal therapy. Overall and post-recurrence survivals were significantly longer in the patients with the amplification [Breslow $p=0.03$ respective $p=0.03$].

In study IV, it was demonstrated that the androgen receptor gene amplification can also occur after antiandrogen monotherapy. It was also shown that combined androgen blockade was effective as a second-line, or in this case, as a third-line therapy, in a patient recurring and showing an amplification of the AR gene.

Study V was a prospective study with seventy-seven patients to investigate whether AR gene amplification could have a role in predicting which patients will benefit from a combined androgen blockade as a second-line hormonal therapy. It was found that patients with the amplification indeed responded better to a second-line combined

therapy according to a decline in PSA as well as palliation of the symptoms. However, association with the prostate cancer specific survival was not found.

In conclusion, this thesis shows that primary prostate cancer is highly treatable with androgen deprivation therapy and decline in PSA during the first year is an independent prognostic factor. Only small fraction of the primary cancers does not respond to hormonal therapy. Measuring the apoptotic index at the time of diagnosis could possibly be useful in identification of endocrine-treated patients with poor prognosis. An androgen receptor gene amplification is found in about 30% of patients having local recurrence after endocrine-therapy. The amplification can also occur after antiandrogen monotherapy, and the amplification predicts a better response to a second-line combined androgen blockade.

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ACKNOWLEDGEMENTS

REFERENCES

ORIGINAL COMMUNICATIONS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following articles, referred in the text by Roman numerals.

I Palmberg, C., Koivisto, P., Visakorpi, T., and Tammela, T. L. (1999); PSA Decline Is an Independent Prognostic Marker in Hormonally Treated Prostate Cancer. Eur.Urol. 36[3], 191-196.

II Palmberg, C., Rantala, I., Tammela, T.L.J., Helin, H. and Koivisto, P.A. (2000); Low apoptotic activity in primary prostate carcinomas without response to hormonal therapy. Oncol. Rep. **7**(5): 1141-1144.

III Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP. (1997); Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res.* 57: 2.314-319.

IV Palmberg C, Koivisto P, Hyytinen E, Isola J, Visakorpi T, Kallioniemi OP, Tammela T. (1997); Androgen receptor gene amplification in a recurrent prostate cancer after monotherapy with the nonsteroidal potent antiandrogen Casodex (bicalutamide) with a subsequent favourable response to maximal androgen blockade. *Eur. Urol.* 31**:** 2.216-219.

V Palmberg, C., Koivisto, P., Kakkola, L., Tammela, T.L.J., Kallioniemi, O-P. and Visakorpi,T. (Dec. 2000) Androgen receptor gene amplification at the time of primary progression predicts response to combined androgen blockade as a second-line therapy in advanced prostate cancer. J. Urol. (in press)

ABBREVIATIONS

ADT	Androgen deprivation therapy
AI	Apoptotic index
ALP	Alkaline phosphatase
AR	Androgen receptor (gene)
ARE	Androgen-responsive element
BM	Bone marrow
bp	Basepair
BRT	Beam radiation therapy
CaP	Carcinoma of prostate / Prostate cancer
CAB	Combined androgen blockade
CPA	Cyproterone acetate
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
EGF	Epidermal growth factor
ER	Estrogen receptor
EVAP	Electrovaporisation of the prostate
FDA	Food and drug administration
FISH	Fluorescence in situ hybridisation
FSH	Follicle-stimulating hormone
FT-PSA	Free to total PSA ratio
hGK-1	Human glandular kallikrein-1
hsp-90	Heat-shock protein 90
IAS	Intermittent androgen suppression
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
ISEL	In-situ end-labelling
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
MAB	Maximal androgen blockade
MRI	Magnetic resonance imaging
NPCP	National Prostate Cancer Project
hPAP	Human prostatic acid phosphatase
PI	Proliferating index

PR	Progesterone receptor
PRL	Prolactine
PSA	Prostate specific antigen
PSAD	PSA- density
PSPA	Performance Status – Pain – Analgesic score
RA	Retinoic acid
RAMBA	Retinoic Acid Metabolism Blocking Agents
SPF	S-phase fraction
SHBG	Steroid hormone binding globuline
SSCP	Single stranded conformation polymorphism
5-α-R	5-α-reductase
T	Testosterone
TRK	Tyrosine kinase
TRUS	Transrectal ultrasound
TURP	Transurethral electroresection of prostate

INTRODUCTION

Prostate cancer [CaP] is the most common male malignancy in the Western world. In Finland 2839 new cases of CaP were diagnosed in 1997, representing an annual age-adjusted incidence of 71.2 per 100 000 men (Finnish Cancer Registry, 2000). The incidence of the disease has rapidly increased during the last two decades, first linearly, then exponentially. This is believed to be mostly due to the use of prostate specific antigen [PSA] even as a screening tool for CaP (Jacobsen et al., 1995; Potosky et al., 1995; Stephenson et al., 1996). In addition, other new diagnostic facilities, e.g. transrectal ultrasound [TRUS] and magnetic resonance imaging [MRI] with endorectal coil, have improved the detection and staging of CaP (D'Amico et al., 1995; Larkin et al., 1986; Lee et al., 1989; Riemenschneider et al., 1989; Salo et al., 1987; Torricelli et al., 1999).

The etiology of CaP is poorly known. As in generally the case in malignancies, environmental factors are considered to be the most important causes also of CaP (Lichtenstein et al., 2000). However, specific environmental factors such as dietary compounds associated with the development of CaP are poorly known. (Armstrong and Doll, 1975; Coughlin et al., 1996; Delfino et al., 1998; Ekman et al., 1999; Kapur, 1999; Schuurman et al., 1999). There is however evidence to indicate e.g. that supplementation of vitamin E [α-tocopherol] as well as a high intake of selenium reduce the risk of CaP (Heinonen et al., 1998; Yoshizawa et al., 1998). The strongest known risk factor for CaP seems to be a positive family history (Gronberg H et al., 1998; Steinberg et al., 1990). One first-degree relative with CaP increases the risk of a man developing the condition 2 to 5 times (Andersson et al., 1996; Bratt et al., 1999a). The familial accumulation of malignancies is considered to be an indication of a hereditary disposition of cancer. Indeed, twin studies have suggested that heritable factors are more important in CaP than in most other common malignancies (Lichtenstein et al., 2000; Verkasalo et al., 1999; Ahlbom et al., 1997). The development of 5-10% of all CaPs is thought to be underlain by mutation in high-penetrance susceptibility genes (Carter et al., 1992). In addition, low penetrance polymorphisms in genes such as the androgen receptor [AR], 5-α-reductase [5-α-R] and vitamin D receptor genes have also been held to be associated with a risk of CaP (Akalu et al., 1999; Ma J et al., 1998; Reichardt et al., 1995). As a conclusion, the etiology of CaP is multifactorial, although androgens, and perhaps even other steroid hormones, are identified as strong tumor promoters (Bosland, 2000).

The prognosis of CaP depends on the stage and the age the disease is diagnosed. Localized CaPs are curable by surgery or external or internal radiation therapy. For incidentally found small cancers with low Gleason score, the prognosis seems to be good regardless of the therapy chosen (Berner et al., 1999; Chang et al., 1991). However, a Finnish study has clearly shown that an incidental carcinoma with diffuse spread in the prostate gland is even more aggressive than a solitary T1-T2 tumour (Haapiainen et al., 1986a). There is no cure for CaP diagnosed at an advanced stage, with either locally invasive tumour or with metastases. This thesis focuses on research into the biology of CaP, especially the androgen sensitivity of new-diagnosed and recurrent tumours, and aims to identify markers that could be used to find ‘the right treatment for the right patient'.

REVIEW OF THE LITERATURE

1. Androgens and growth of prostate cancer

1.1. Androgens

Androgens are mainly secreted by testicular Leydig cells. The testes produce primarily testosterone [T], but also androstenedione and dihydrotestosterone [DHT], which is the active metabolite of testosterone. In addition to the testis, 5-10% of the androgens is produced by adrenal glands as dehydroepiandrosterone [DHEA], its sulfate dehydroepiandrosterone sulfate [DHEA-S] and androstenedione. In the prostate gland, DHT is converted from T by enzyme 5-α-reductase (Russell and Wilson, 1994). DHT is also produced in small amounts by reductive 3-α-hydroxysteroid dehydrogenase. Androgen production requires a pulsatile stimulus by LH-FSH from the pituitary gland. After castration the serum level of T decreases by 90-95%, while the intracellular level of DHT decreases by only 50-60% (Geller, 1985; Labrie et al., 1993).

Androgens control the development and differentiation of the male reproductive organs (Cooke et al., 1991). It is known that androgens exert effects on regulation of the expression of numerous genes and their products, e.g. tumour markers PSA, human prostatic acid phosphatase [hPAP] and human glandular kallikrein-1 [hGK-1] (Perry et al., 1996; Young et al., 1992).

The majority of CaPs arise from the secretory, androgen-dependent glandular epithelial cells. Castration of a male before puberty inhibits the growth of the prostate and prevents the initiation of CaP (Isaacs, 1994; Moore, 1944). Thus, androgens are believed to have, at least, a permissive role in the genesis of CaP.

1.2. Androgen receptor

Androgen action at the target organ is mediated via specific nuclear receptors (Lubahn et al., 1988). The androgen receptor [AR, Figure 1] gene is located in chromosome X between the centromere and band q13 (Kuiper et al., 1989). The gene contains eight exons, designated from A to H (Lubahn et al., 1988). The total

length of the gene is 90 000 basepairs [bp] (Kuiper et al., 1989). Like other steroid receptors, AR consists of amino-terminal transactivation [exon A], DNA- binding [exons B and C] and hormone- binding domains [exons D-H].

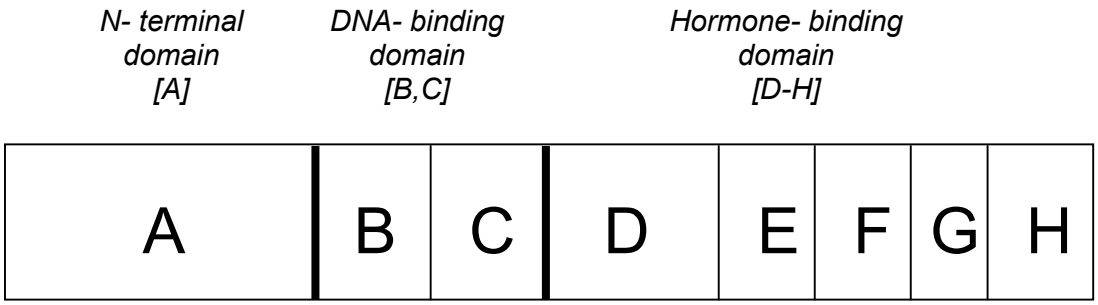

Figure 1: The schematic structure of the androgen receptor. Exon A contains polymorphic repeats of glutamine (CAG_n) and glycine (GGC_n).

The mechanism of function of AR-mediated transcription is fairly well known [Figure 2]. The cascade of activation is initiated by the binding of a ligand to the hormone-binding domain (Henttu et al., 1992). In the absence of ligand, AR is thought to interact with hsp-90 and p59, preventing the binding of AR to DNA (Baulieu et al., 1990). When bound to ligand, hsp-90 and p59 are released and the new receptor-ligand complex binds to specific androgen responsive elements [AREs] on DNA in the 5'-prime region of the target genes. Transcriptional activation by AR involves formation of a multiprotein complex, including a number of newly cloned AR co-regulators (Koivisto et al., 1998)

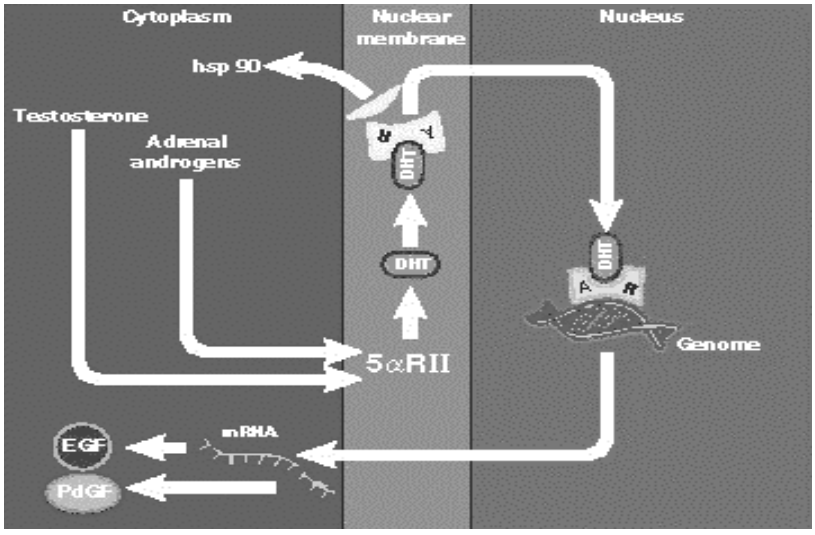

Figure 2: Androgen action in the prostate gland

1.2.1. *Androgen receptor in prostate cancer*

The hyperplastic prostate shows constant expression of AR in glandular cells, while stromal cells show only weak expression (Chang and Chung, 1989; Chodak et al., 1992). Coffey and Isaacs suggested that the content of AR in tumorous tissue determines whether it was an androgen-dependent or independent disease (Coffey and Isaacs, 1981) in similar fashion as in breast cancer the expression of estrogen [ER] and progesterone [PR] receptors predicts hormone sensitivity of the disease (Helin et al., 1989; Helle et al., 1988). However, modern AR immunohistochemistry has now shown that both hormone-naïve and refractory prostate tumours express AR (Hobisch et al., 1996; Ruizeveld de Winter et al., 1994; Visakorpi et al., 1995).

Structural alterations in the AR have been extensively investigated in CaP. Studies on germ-line alterations have indicated that men with short CAG-repeat length in the first exon of the AR gene run an increased risk of CaP (Giovannucci et al., 1997; Stanford et al., 1997). However, there are also studies claiming that CAG-repeat length is not associated with CaP risk (Bratt et al 1999b; Correa-Cerro et al., 1999; Edwards et al., 1999; Sartor et al., 1999). In radically treated CaP it has been suggested that a short CAG-triplet repeat may be important for CaP recurrence among patients who are otherwise considered as low-risk patients (Nam et al., 2000).

It was recently shown in a Finnish study that germ-line alteration in codon 726 [Arg->Leu] is found in 2% of CaP patients, whereas in the general population this alteration is found in only 0.5%. The findings suggest that this germ-line mutation could increase the CaP relative risk by 4.4 (Mononen et al., 1999). It has previously been shown that the codon 726 [Arg->Leu] mutation alters the transactivational properties of the receptor (Elo et al., 1995).

There are several studies that have screened acquired mutations in prostate tumours. The majority of these have indicated that mutations are rare in untreated, new-diagnosed CaP, but can occur at an advanced stage of untreated CaP (Koivisto et al., 1998; Marcelli et al., 2000). However, two studies have suggested that mutations could be found in as many as 25-45 % of the untreated CaPs (Gaddipati et al., 1994; Tilley et al., 1996). The discrepancy in the observed mutation frequencies could be due to methodological aspects, or to the possibility that tumours believed to be from untreated patients were actually obtained from treated patients.

Fewer studies have screened hormone-refractory CaPs. However, it would seem that the mutations in question are rare in tumours treated by castration (Elo et al., 1995; Evans et al., 1996; Wallen et al., 1999). On the other hand, it has been reported that the codon 877 [Thr->Ala] mutation is found in 30-50% of flutamide-treated patients, suggesting that the treatment selects the mutation (Taplin et al., 1995 and 1999). The same mutation has been found in an LNCaP CaP cell line; moreover the mutation alters the transactivational properties of the receptor in such a manner that it can be activated paradoxically by flutamide (Veldscholte et al., 1990). Recently, it has also been shown that a double mutation in the AR leads to AR function as a high-affinity cortisol / cortisone receptor (Zhao et al., 2000). The frequency of such mutation is, however, probably quite low.

Certain mutations in the AR seem to be able to alter the transactivational properties of the receptor as described above. In addition, it has been suggested that in the presence of only low levels of androgens other growth factors, such as EGF and IGF-1, could activate the receptor (Culig et al., 1994; Jenster 2000). Cross-talk between HER-2/neu and AR signalling pathways has moreover been demonstrated in cell lines and xenograft models (Craft et al., 1999; Yeh et al., 1999). The true significance of these alternative modes for AR activation obviously calls for more detailed study.

Visakorpi and co-workers have identified an amplification of the AR gene in about one third of locally recurrent, hormonally treated CaP tissue samples (Visakorpi et al., 1995). This finding indicated a new mechanism of therapy failure in hormonally treated CaP. It also suggested that the hormone-refractory CaPs are perhaps not androgen-independent, but instead hypersensitive to low circulating levels of androgens.

1.3 Programmed cell death [apoptosis] and cell proliferation

The theory underlying androgen ablation as a treatment for CaP is based on the androgen dependency of prostatic gland growth. Castration initiates a cascade called programmed cell death, or apoptosis (Isaacs, 1984; Kyprianou et al., 1990). Normal prostatic glandular cells undergo apoptosis with approximately 20% cell death daily two to three days from castration (Berges et al., 1993). Castration also induces apoptosis in cancer cells. However, the apoptotic response seems to differ from one tumour to another (Westin et al., 1995). Androgen withdrawal initially reduces the cell proliferation rate in both benign and malignant prostatic tissue (Denmeade et al.,

1996; Isaacs et al., 1992; Westin et al., 1995). Further, it has been suggested that the effects of castration may be mediated even more by reduced proliferation than by increased apoptosis (Westin et al., 1995).

1.4. Androgen sensitivity of prostate cancer

CaP is probably the most markedly hormone-dependent cancer in man, and it does not exist in men castrated in pre-puberty (Isaacs, 1994; Moore, 1944). Huggins and Hodges showed as far back as in the 40's that nearly all CaPs are androgen-dependent at the time of diagnosis, and androgen deprivation has since been the cornerstone of therapy for CaP (Huggins et al., 1941). The response rate for castration has been reported to be between 60-90%, but most tumours relapse after 12 to 18 months (Grayhack et al., 1987; Mahler and Denis, 1992).

A recurrent CaP after androgen deprivation therapy [ADT] has been considered to be hormone-refractory, or to represent the androgen-insensitive / -independent, phase of the disease. It has nonetheless been suggested that patients do indeed benefit from continued ADT despite the relapse (Chao and Harland, 1997; Taylor et al., 1993). In other words, it is believed that CaP is still under some hormonal control even if regarded as 'hormone-refractory'. It has also been demonstrated that administration of T to patients with 'hormone-refractory' CaP aggravates disease symptoms (Denis and Nowe, 1980; Labrie et al., 1993).

The mechanisms leading to a relapse under hormonal therapy of CaP are poorly understood. A number of different hypotheses have recently been presented following improvement in techniques in molecular biology. Two basic concepts for the development of hormone-refractory CaP have been suggested: adaptation and clonal selection. In rodent models it has been shown that androgen-dependent CaP cells may develop secondary genetic mutations which allow androgen-independent growth, indicating tumour adaptation to a low level of androgens (Klein et al., 1997). Alternatively, it has been suggested that hormone-refractory tumour cells may already exist in the early stage of CaP, and that castration gives a growth advantage to the hormone-refractory clone. The clonal selection theory is supported by the intratumour heterogeneity found in the early-stage CaP (Jenkins et al., 1997). In addition, it has been shown that castration may select a tumour clone having genetically very different content from that in the hormone-naïve tumour (Nupponen et al., 1998). Recently, Craft and associates (1999) also showed by propagating

androgen-dependent LAPC-9 xenograft in castrated male mice that the androgen-insensitive cells were present in the xenograft at a frequency of 1 to 10^5-10^6 androgen-dependent cells. Further, the castration led to outgrowth of the hormone-refractory cells.

At a molecular level, androgen–insensitive progression has been associated with several genes and signal transduction pathways. The role of the AR signalling pathway has already been dicussed above. Other putative molecular mechanisms include activation of c-myc oncogene, or anti-apopototic bcl-2 (Krontiris, 1995; McDonnell et al., 1992).

2. Therapy of prostate cancer

The natural course of CaP is highly variable and difficult to predict. In the modern era of prostate specific antigen [PSA] and TRUS, over 80% of newly diagnosed CaPs are still organ-confined at the time of diagnosis (Freedland et al., 2000; Määttänen et al., 1999). At this stage, CaP is theoretically curable. It has even been suggested that active watchful waiting might suffice for patients with low-grade small organ-confined growths (Feneley, 1999; Johansson et al., 1997). Once the CaP has invaded areas outside the prostate gland it is no longer curable, and only palliative treatment modalities are available.

2.1. Radical therapy

Radical therapy of CaP, or treatment with intention to cure, can be achieved mainly by two means: surgery or beam radiation therapy [BRT]. The surgical approach, i.e. radical prostatovesiculectomy, has become a routine procedure for urologists, and due to improved operation techniques even the intraoperative and postoperative mortality and morbidity have decreased markedly (Mark, 1994; Walsh et al., 1988 and 1994). The operation is performed mostly on younger patients with active sexual functions. Thus, the technique introduced at the beginning of the 1980's by Walsh which preserves neurovascular bundles and the capacity for penile erection after the operation, made this therapy option more popular (Walsh et al., 1983). The most common long-term adverse effects of radical surgery are impotence [30-40%] and incontinence [5-15%] (Catalona et al., 1999; Quinlan et al., 1991; Steiner et al.,

1991; Walsh et al., 1994). Despite 'a radical procedure', according to preoperative investigations and pathological investigation of specimen, 30% of patients in fact experience a relapse of CaP via elevated PSA or locally or distant recurrent disease (Walsh et al., 1994).

BRT is applied as a curative mode externally or internally [brachytherapy] with radioactive seed implants. The results of external BRT reported in some studies are as good as those of surgery (Martinez et al., 2000; Shipley et al., 1999). External BRT is also recommended, with or without adjuvant therapy, for patients with locally advanced disease, or for patients too old for radical surgery (Akakura et al., 1999a and 1999b; Aro et al., 1988; Stromberg et al., 1997). On the other hand, brachytherapy is feasible for small tumours and for patients with wish of preserved potency (D'Amico et al., 1996). Especially for patients with 'low-risk disease' [i.e. Gleason score <6, T1c or T2, PSA < or =10] brachytherapy gives as good prognosis as radical prostatectomy or external BRT (D'Amico et al., 1998). For BRT the most common late adverse effects are irritation of the urinary bladder and rectum, enteritis, urethral stricture, incontinence and impotence. The irritative adverse effects are dose-dependent and occur in about 5% of the patients (Shipley et al., 1994).

Other, less commonly used radical treament forms include ultrasound-guided percutaneous cryoablation (Bahn et al., 1995; Lee et al., 1994) and high-intensity focused ultrasound (Beerlage et al., 1999; Chapelon et al., 1999; Madersbacher et al., 1995).

2.2 *First-line hormonal treatment*

Hormonal therapy was first introduced in clinical practice in the mid-40s. In 1941 a study published by Huggins and Hodges showed perceptible clinical improvement after removal of androgens in 18 out of 21 patients [86%] with advanced prostatic carcinoma (Huggins et al., 1941). Hormonal therapy is still the cornerstone in the treatment of locally advanced or metastasized CaP; 60-90% of patients are said to respond to hormonal treatment as primary therapy (Grayhack et al., 1987; Mahler and Denis, 1995), but the therapeutic effect often decreases and the disease relapses after 12-18 months (Mahler and Denis, 1992; Newling, 1996).

2.2.1. Surgical and chemical castration

The most common form of hormonal therapy is castration, achieved by surgical or chemical means. Orchidectomy is a simple surgical procedure which eliminates the circulating androgens by 95% within 2-6 hours, and gives rapid pain relief (Bergman et al., 1982; Maatman et al., 1985). A modern and non-surgical mode of castration is the use of subcutaneous injections of luteinizing hormone-releasing hormone [LHRH] analogues, which discontinues the pulsatile fashion the hormone is normally secreted by the hypothalamus (Auclair et al., 1977). The continuous secretion of LHRH first increases the secretion of luteinizing hormone [LH], leading to a phenomenon called 'flare'. This usually exacerbates symptoms such as pain and may even lead to paraplegia and death (Parmar et al., 1985; Thompson et al., 1990). In three to four days, the absence of pulsatile stimuli for Leydig cells in the testes results in diminished production of testosterone, i.e. chemical castration (Auclair et al., 1977). Treatment with LHRH analogues is a feasible and potent means of treating CaP, but the cost of the injections is high. The most common side-effects of castration are loss of libido and impotency, sweating as well as slight feminization, even osteoporosis as a late effect (Daniell, 1997; Parmar et al., 1985). Chemical castration can also be chosen as a neoadjuvant therapy before therapy-with-intention-to-cure or as an adjuvant therapy after radical treatment by surgery or radiation therapy (Bolla et al., 1997; Meyer et al., 1999)

2.2.2. Estrogens

Estrogens were previously widely used as the first line of therapy in advanced CaP. The main action of estrogens is suppression of the release of LH and follicle-stimulating hormone [FSH] from the pituitary gland, leading to a decrease in androgens to castration level. Estrogens also have a direct effect at cellular level on the prostatic cells, and the hormone stimulates osteoclast activity in the bones and increases the level of steroid hormone-binding globulines [SHBG] and prolactine hormone [PRL] in the serum (Haapiainen et al., 1986b). The former hormonal effect lowers the amount of circulating androgens in blood, and the latter has a direct negative effect on the prostate gland. The most commonly administered estrogen is diethylstilbestrol [DES] enterally, which at a dose of 1 mg has been shown to have an effect on serum testosterone level equivalent to that of surgical castration (Beck et al., 1978). Treatment with estrogens is a cheap alternative, but because of the cardiovascular adverse effects of oral estrogens their use has decreased

dramatically (Byar, 1980). In Scandinavia, the most commonly used estrogen is parental polyestradiol phosphate, which has now been shown not to increase cardiovascular mortality (Aro et al., 1991; Hedlund and Henriksson, 2000).

2.2.3. Antiandrogens

Antiandrogens, steroidal and non-steroidal, represent a unique group of medicines used to treat advanced CaP or relapsed disease after therapy with radical intention. These drugs can be used as monotherapy, neo-adjuvant therapy prior to radical surgery or BRT, or in combination with castration therapy (Debruyne et al., 1994; Di Silverio and Sciarra, 1986; Geller and Albert, 1985; Laverdiere et al., 1997; Soloway, 1984).

The only steroidal antiandrogen in use in Finland is cyproterone acetate [CPA], which has a double function in its central progestine and anti-gonadotropic effect and competitive peripheral blocking of androgen receptors at target organ (Barradell and Faulds, 1994). The central effect is often temporary, and after 6 to 12 months its antiandrogenic effect decreases (McLeod, 1993). The adverse effects of CPA are more or less the same as those of castration therapy (de Voogt, 1992). The non-steroidal, pure antiandrogens [flutamide, nilutamide and bicalutamide] have reduced the use of CPA because they have no central effects. For example, when used as monotherapy, the non-steroidal antiandrogens may preserve libido and potency in younger CaP patients. This, however, was not fully confirmed in a recent European study of 310 CaP patients (Schröder et al., 2000). In patients treated with non-steroidal antiandrogens as monotherapy, the serum level of circulating androgens increases by 40-60% (Balzano et al., 1987). Adverse effects of non-steroidal antiandrogens arise mostly from the gastro-intestinal tract, i.e. diarrhea, but also breast tenderness and gynecomasty (Sarosdy, 1999). The price of the therapy with non-steroidal antiandrogens is approximate that of LHRH agonists.

2.2.4. Maximal or combined androgen blockade

Maximal, or combined, androgen blockade [MAB or CAB] is a therapy option which provides blockade of not only testicular but also adrenal androgens. In CAB therapy,

surgical or chemical castration is combined with nonsteroidal antiandrogen to obtain maximal, but obviously not total blockade of androgen receptors at the target organ.

This therapy modality was first introduced in the 1940's as adrenalectomy combined with orchidectomy (Huggins and Scott, 1945). The adverse effects were, however, fatal by reason of adrenal insufficiency. Labrie and co-workers re-introduced the treatment option in 1983 as primary treatment in a study of 37 newly diagnosed patients treated with LHRH agonist and flutamide in combination (Labrie et al., 1983). In a larger study of 603 patients by Crawford and associates in 1989, CAB therapy was shown to be superior to treatment with LHRH analogue alone (Crawford et al., 1989). In a European study by Denis and colleagues CAB was also shown to give a longer median survival time of 34.4 vs. 27.1 months (Denis et al., 1993). In later studies this has, however, not been confirmed. In meta-analyses of 22 and 27 randomized studies, there were no significant differences in survival between patients treated with either CAB or monotherapy (Laufer et al., 2000; Prostate Cancer Trialists' Collaborative Group, 1995) Above all, in a large study of 1387 patients randomized to receive either orchiectomy alone or in combination with flutamide, no benefit was found in comparison CAB (Eisenberger et al.,1998).

2.2.5. Intermittent androgen supression [IAS]

The main problem in the conservative regimen of hormonal treatment is that the cancerous tissue becomes increasingly less sensitive to the therapy and the disease relapses. There are several ongoing studies concerning intermittent hormonal therapy, (Crook et al., 1999; Goldenberg et al., 1995; Klotz et al., 1986). The rationale behind IAS is to achieve a prolongation of the androgen sensitivity of CaP tissue and by that means to delay relapse of CaP.

2.3. Relapsed disease - second-line therapy

2.3.1. Relapse after radical treatment

When CaP relapses after radical treatment the therapy options are the same as for advanced CaP. The critical question is, however, when to commence treatment. It is widely accepted that an adjuvant treatment is best initiated early, if the disease is not organ-confined and PSA does not fall to undetectable level after the operation

(Messing et al., 1999; Valicenti et al., 1999). In local relapses external BRT is one appropriate therapy option, whereas in the case of positive lymph nodes the therapy should be systemic (Messing et al., 1999; Valicenti et al., 1999).

2.3.2. Relapse after hormonal treatment

When hormonal therapy fails the prognosis is poor (Fosså, 1994). The median survival time for CaP patients with bone metastases whose disease relapses during primary androgen deprivation therapy is 9-12 months (Blumenstein et al., 1993; Fournier, 1996).

2.3.2.1. *Combined androgen blockade*

When CaP starts to progress, addition of a non-steroidal antiandrogen to surgical or chemical castration may be of benefit. However, only about 30% of patients with second-line CAB benefit from the therapy (Labrie et al., 1988). The problem seems to lie in finding the right patients who will derive benefit from the treatment.

2.3.2.2. *Estramustine phosphate*

Estramustine phosphate, nor-nitrogen mustard linked to estradiol-17β-phosphate, is the cytotoxic agent most commonly used by urologists in Finland in relapsed CaP. It was introduced by I. Könyves in the 1960's and the first clinical study on advanced, hormone-refractory human CaP was presented by Szendröi and associates (1974). The rationale for the chemical attachment of a mustard moiety [an alkylating agent] to the estrogen was targeting of the cytotoxic agent against cancerous tissue via steroid hormone receptors (Tew et al., 1992). However, the most effective anti-mitotic activity of the medicine derives from the estramustine phosphate metabolites estromustine and estramustine, which disturb the microtubular organization in a CaP cell (Hartley-Asp, 1984). This anti-mitotic activity could be of benefit prior to external BRT of CaP as a radio-sensitizing drug (Eklöv et al., 1994; Schmidt et al., 1993). An objective response rate of 20-40% has been reported for patients relapsing during primary hormonal therapy, and in a subgroup of patients the effect was even better (Iversen et al., 1997; Morote et al., 1991).

The most common adverse effects of estramustine phosphate are dose-dependent, nausea and gastrointestinal disorders, but seldom myelosuppression (Perry and

McTavish, 1995). This has to be borne in mind when concomitant administration with other therapeutic agents, e.g. clodronate, occurs (Kylmälä et al., 1996).

2.3.2.3 *Other cytotoxic therapy*

When primary hormonal therapy fails, the patients are assumed to have hormone-insensitive, or refractory, disease, and further hormonal manipulation has been found to elicit only a short-lived response in one subgroup of patients. Cytotoxic forms of therapy remain as theoretical options. However, the results reported after a variety of such treatments with either single-agent or combination therapy are poor, with response rates between 15-30% (Fosså and Paus, 1994). Recently improved results have been reported with fosfestrol, which yielded a good PSA-based response rate for 31 out of 39 patients [79%] (Orlando et al., 2000).

2.3.2.4 Other current medical treatments

Ketoconazole, an oral imidazole derivative with antifungal properties, has been shown to inhibit both adrenal and testicular androgen synthesis (Rajfer et al., 1986). One possibility to treat hormone-relapsed CaP is with an anti-parasitic agent, suramin, which has the ability to bind and to inactivate growth factor and enzyme systems critical to cellular homeostasis and proliferation (LaRocca et al., 1991).

2.3.3 *New experimental therapy options*

Ongoing research is warranted to find new solutions in the treatment of hormone-refractory CaP. The prognosis for such relapsed patients has not improved during the last four decades (Blumenstein et al., 1993; Fournier, 1996). A better understanding of the molecular and cell biology of CaP will make for new definitions of targets of therapy.

- *Activation of programmed cell death* [apoptosis]: In hormone-insensitive CaP androgen withdrawal does not lead to apoptosis of CaP cells. Apoptosis can be activated pharmacologically e.g. by pro-thapsigargine, which is activated at the tumour site by PSA (Furuya et al., 1994). This leads to an increase in Ca^{++} in the cell and activation of apoptotic mechanisms. A pro-drug is needed on account of the system toxicity of thapsigargine administration.

- *Inhibition of signal transduction:* CaP cells can switch from paracrine to autocrine control of cell growth. This is mediated by various neurotrophins, and by tyrosine kinase [TRK] receptor phosphorylation. A specific TRK inhibitor called RG-13022 has been developed and shown to have a growth-inhibiting effect on androgen-independent cell lines (Kondapaka and Reddy, 1996).
- *Deactivation of telomerase activity:* Telomeres protect chromosomes at both ends, and are shortened after each somatic cell division. This leads towards an apoptotic, programmed, death of the cell. Cancerous cells can reactivate or upregulate telomerase activity and thus become 'immortal' by preservation or new synthesis of telomeres (Sommerfeld et al., 1996).
- *Differentiation therapy:* Loss of histological differentiation is associated with the progression of CaP. Other intercellular or intracellular mechanisms involved in its progression are loss of E-cadherin, which mediates the information keeping like cells alike together (Mareel et al., 1993), and decreased intracellular levels of retinoic acid [RA] and vitamin D (Blutt et al., 1997). Therapy with vitamin D-analogues and retinoic acid metabolism blocking agents [RAMBA], e.g. liarozole, involves adaptation of cytochrome P450-dependent metabolism, dependence on the presence of nuclear receptors and possible side-effects in clinical use (De Coster et al., 1992). RA has been shown to have an in vitro anti-tumour effect on e.g. Dunning CaP cell lines, and an in vivo anti-proliferative effect on human CaP cell lines (Dijkman et al., 1994; Seidmon et al., 1995). It also leads to increased expression of E-cadherin and the histological differentiation of CaP cells becomes more squamous (Smets et al., 1995).
- *Gene therapy:* This therapy option is gaining ground in phase II trials. At least 20 clinical trials have been reviewed by FDA. A number of strategies have been utilized, for example immunomodulation by IL-2, restoration of missing tumour suppressor gene p53 activity and so-called suicide gene therapy with thymidine kinase gene (Hassan et al., 2000; Hrouda D et al., 1999; Shalev at al.,2000)

2.3.4. Late-stage palliative procedures

In the late stage of CaP the most important issue is to make life as comfortable as possible for the patient (Labasky and Smith, 1988). Pain due to skeletal metastases, uremia due to prostatic enlargement or cancer infiltration of the urinary bladder has to be taken care of. Methods of killing the pain, in addition to non-steroidal anti-inflammatory drugs or opioids, are external BRT to the pain lesion and / or

radioactive isotopes, e.g. strontium or samarium, (Houston and Rubens, 1995; Malmberg et al., 1997), and bisphosphonates (Coleman, 1998). However, according to a Finnish study of 57 patients by Kylmälä and associates (1997) clodronate in combination with estramustine phosphate is not sufficient to ease the pain due to skeletal metastases.

Uremia and hydronephrosis are best managed with double-pigtail stenting or external pyelonephrostomas (Kohler et al., 1980; Perinetti, 1982). If CaP causes infravesical obstruction or continuous macroscopic hematuria it might be useful to interfere by using a palliative transurethral electroresection of the prostate [TURP] or electrovaporization of the gland [EVAP] (Mora Durban et al., 1995). There are several studies from the 80's which show that TURP has an adverse effect on prognosis (Kuban et al., 1987; Levine et al., 1986), but also that there is no correlation between TURP and the probable dissemination of the disease (Schwemmer et al., 1986). According to a study by Trygg and colleagues (1998) it was mainly concomitant diseases and not the TURP itself which worsened prognosis for a CaP patient.

3. Prognostic markers of prostate cancer

3.1. *Prostate specific antigen*

Since the identification of prostate specific antigen [PSA] by Ablin and group in 1970, and the purification of the enzyme from prostatic tissue by Wang and colleagues (1979) it has been the most widely used and most accurate tumour marker in man. Its physiological function is to dissolve the gel in freshly ejaculated semen (Stenman et al., 1999). The synthesis and production of PSA is strongly dependent on androgens, although other factors such as retinoic acid and growth factors also increase PSA production (Seregni et al., 1996). Despite its name it is also produced in small quantities by breast tumours and tumours of the thyroid gland (Alanen et al., 1999; Ro et al., 1994). Normal prostatic tissue produces PSA at 0.15-0.20 ng/ml/g tissue (Lee and Littrup, 1992; Lee et al., 1992). The half-life of PSA in serum is about two-and-a-half to three days (Ravery et al., 1998). In a case of CaP, PSA is believed to leak into the serum due to morphological changes in prostate cells and due to a greater concentration of the protein in tumour cell cytosol, leading to an

increase in the serum concentration of PSA (Catalona et al., 1991; Culkin et al., 1995). Most of the PSA is in complex form. It has recently been shown that the specificity of PSA in detecting CaP can be increased by measuring the free to total PSA ratio [FT-PSA] (Lilja et al., 1991; Stenman et al., 1991; Lilja and Stenman, 1996). Previously the velocity and the density of PSA was often calculated, but the velocity of PSA is difficult to monitor, and subsequent studies of prostate specific antigen density [PSAD] showed that it does not provide additional information in detecting CaP (Raviv et al., 1996).

After radical surgery, i.e. prostatovesiculectomy, PSA decreases to an 'undetectable' level after only a couple of weeks from the operation, as a sign of complete removal of normal and malignant prostate cells (Brandle et al., 1999; Partin et al., 1996). According to Vassilikos and colleagues (2000), an increase in PSA [detection limit 0.0001 ng/ml] by ultrasensitive assay predicts ‘conservative biochemical relapse’ within at least 18 months. Clinical relapses are accompanied by a rise in PSA and the elevation of PSA precedes clinical relapse by about fifteen months (Kupelian et al., 1996). In relapse after radical prostatovesiculectomy with PSA progression the prognosis is held to be poorer for patients with a high percentage of FT-PSA (Wojno et al., 1998).

After hormonal therapy, i.e. androgen withdrawal, the level of PSA decreases fairly rapidly. The decrease is caused by the lack of androgens, and to second by the diminishing tumour burden (Kirschenbaum et al.,1996; Nevalainen et al., 1993; Oesterling et al., 1993; Roehrborn et al., 2000). According to several studies the velocity and the nadir of PSA decline after the initiation of androgen deprivation therapy plays a prognostic role in CaP (Arai et al., 1990; Miller et al., 1992; Oosterlinck et al., 1997). In monitoring the disease outcome after therapy PSA does not tell whether the relapse is local or metastatic; however, it precedes the clinical relapse often by months (Newling, 1993). Even after relapse of first-line hormonal therapy PSA decline has a prognostic role when initiating second-line therapy (Matzkin and Soloway, 1992).

3.2. *Alkaline phosphatase*

Alkaline phosphatase [ALP] is also used both in monitoring disease outcome and as a prognostic factor. The normal range of ALP in males is 60-275 U/ l. ALP increases both in biliary diseases and if skeletal metastases appear. Measurement of ALP iso-

enzymes improves accuracy in determining the possibility of skeletal metastases (Cooper at al., 1994; Desoize et al., 1991). It has been suggested that ALP indicates the prognosis even better than PSA (Mackintosh et al., 1990).

3.3. *Estrogen and testosterone*

High level of serum testosterone is considered to be a risk factor for prostate cancer (Gittes 1991). In addition, several studies have evaluated prognostic utility of pretreatment serum testosterone as well as estrogen levels. The results have been quite conflicting (Andersson et al., 1993; Carter et al., 1995; Höisäter et al., 1982). However, in a recent Finnish study by Mikkola and group (1999) it was shown that plasma estradiol was significantly higher in M0 than in M1 patients, whereas the serum testosterone level did not differ significantly between groups.

3.4. *Other prognostic factors*

The old and well-known prognostic markers, i.e. the stage of the disease and the differentiation of the tumour, are inadequate in identifying the high-risk patients at an early stage of CaP. Newer markers such as flow cytometric S-phase fraction [SPF], DNA ploidy, tumour proliferation measured by Ki-67 and p53 overexpression may give more accurate information on disease aggressiveness and predict the outcome (Aaltomaa et al., 1997; Berner at al., 1995; Vesalainen et al., 1994; Westin et al., 1995).

For example, DNA ploidy and SPF determined by flow cytometry are independent prognostic factors in CaP (Visakorpi et al., 1991; Visakorpi 1992a; Vesalainen et al., 1994). High SPF seems even to predict poor outcome after relapse (Visakorpi et al., 1994). Another commonly used method for detection of proliferation activity is Ki-67 immunohistochemistry. There are moreover numerous studies indicating that Ki-67 is even an independent prognostic marker in CaP (Ahlgren et al. 1999; Baretton et al., 1999; Stattin et al. 1997)

Isaacs and coworkers were the first group to report of the possible role of p53 in CaP (Isaacs et al., 1991). Subsequently Effert and associates (1992) and Visakorpi and associates (1992b) showed that accumulation of protein p53 was associated with

both the progression of CaP and a poor outcome of the disease. It has since been shown that TP53-gene mutations are most often late events in CaP (Berner et al., 1995). Recent studies by Cheng and colleagues (1999a) suggest that overexpression of p53 in lymph nodes dissected in radical prostatectomy predicts better an aggressive disease than p53 overexpression in the primary tumour. p53 overexpression seems also to be associated with a higher cell proliferation rate after external radiation therapy (Cheng et al., 1999b).

AIMS OF THE STUDY

The overall goal of this present series was to identify prognostic and predictive markers for endocrine-treated CaP.

The special aims of the study were:

1. To estimate the prevalence of primary androgen- insensitive CaP (I)
2. To study the prognostic value of prostatic specific antigen in hormonally treated CaP (I).
3. To investigate markers for differentiation of primary androgen-insensitive and sensitive CaP (II).
4. To evaluate biological and clinical characteristics of CaP involving androgen receptor gene amplification (III).
5. To establish whether AR gene amplification at the time of primary progression predicts response to second-line combined androgen blockade (IV, V).

MATERIAL AND METHODS

1. Patients and samples

1.1. Study I - PSA as a prognostic marker in prostate cancer

This retrospective study involved 236 consecutive patients diagnosed as having CaP and treated in Tampere University Hospital 1990-1994 with androgen deprivation therapy by surgical or medical castration [203 patients] or antiandrogen monotherapy [29 with bicalutamide, three with CPA. One patient was treated with CAB as the first line therapy. Patient files were studied and response to therapy determined as a decline in PSA during the first 12 months of therapy. The following criteria were used for biochemical response categories:

- **complete response (CR):** PSA decline to undetectable level (< 1.0 ng / ml) in at least two measurements, one of which at 12 months.
- **partial response (PR):** PSA decline of more than 90% from pre-treatment level and at least one measurement within the normal range of PSA (0-4 ng/ml).
- **stable disease (SD):** PSA decline of more than 50% from baseline, but not necessarily to normal range.
- **no response (NR):** PSA decrease of less than 50% or increasing level.

1.2. Study II - Primary androgen-insensitive prostate cancer

This retrospective study involved 10 patients with untreated, primary androgen-insensitive CaP and 20 patients with androgen dependent CaP treated by conventional hormonal therapy during the 1980's. All patients were followed until death.

Primary tumour specimens taken before any treatment were available from all patients. Patients were matched to each other by age, grade [WHO] and stage of disease and tissue samples from the prostate taken before therapy [five core biopsies and fifteen TURP specimens in the sensitive group and five core biopsies and five TURP specimens in the insensitive group]. All patients received androgen deprivation therapy by orchidectomy or LHRH analogue [three patients in the insensitive group]. Representative formalin-fixed, paraffin-embedded tumour blocks were selected by histopathological examination of hematoxylin and eosin-stained slides.

1.3. Study III - Biological and clinical characteristics of prostate carcinomas containing AR gene amplification

This retrospective material consisted of 54 hormonally treated CaP patients in whom the cancer had relapsed during primary hormonal therapy and who had a locally recurrent tumour. The patients had symptoms of urinary obstruction and an increase in serum PSA or serum prostatic acid phosphatase (before 1991). Fifty-one of the patients were primarily treated in Tampere University Hospital, three in Dijkzigt Hospital, Rotterdam, the Netherlands. Conventional androgen deprivation therapy consisting of orchidectomy (37 cases), LHRH analogue (6), estrogen (6), or orchidectomy and estrogen (5) was the primary treatment for these patients.

The clinical response to primary therapy was evaluated by NPCP criteria retrospectively from patient files. Paraffin-embedded tumour specimens [TURP] were available in every case after the relapse of the disease, and paired tumour specimens taken before any therapy were available for twenty-six patients [seventeen TruCut biopsies, nine TURP]. These slides were further selected and prepared for interphase FISH.

1.4 Study IV - AR gene amplification after monotherapy with nonsteroidal antiandrogen

The patient here was first treated by bicalutamide monotherapy at 150 mg a day. PSA nadir was reached at four months [19 ng/ml], although the value never reached remission level. At seven months PSA increased slightly to 21 ng/ml, the skeletal scintigram yielded a suspicious finding in one vertebra and the patient had started to complain of urinary disturbances. TURP was performed and he was enrolled in a trial with ifosfamide therapy [1,5g/m^2 i.v. administration during four days with three-week intervals]. The cytotoxic therapy had no effect on CaP, PSA rose [41 ng/ml after six ifosfamide infusions when third-line therapy was introduced], the skeletal scintigram showed new uptake and the general condition was significantly weakened. CAB was introduced with LHRH analogue. The general condition was now markedly improved, the patient gained 10 kg weight and the WHO performance score reverted to 0. The positive effect of CAB lasted for five months.

The clinical outcome was evaluated retrospectively from the patient's files. A TruCut needle biopsy from the prostate used for FISH analyses was taken at the time of diagnosis, and at the time of TURP as a transurethral resection tissue specimen.

1.5 Study V - AR gene amplification as a predictor of response to combined androgen blockade

This prospective study recruited 92 consecutive patients with advanced CaP, whose disease progressed during conventional androgen deprivation therapy. The patients were treated at Tampere University Hospital during the period January 1994 to February 1997. Fifteen were excluded from the study regimen because of absence of cancerous tissue in the prostate biopsies taken at the time of relapse, and 77 were thus included in the analysis. The 15 excluded patients did not differ significantly from the 77 in whom the primary therapy was orchidectomy [54 patients], LHRH- analogue [13] and bicalutamide monotherapy [10].

At the time of relapse, which was defined as elevation of PSA in at least two consecutive measurements with an interval of one to three months, and/or bone pain associated with positive findings in bone scan and/or signs of local recurrence of the disease. Core biopsies were taken from the prostate gland at the time of relapse from first-line endocrine therapy for both pathological analysis and interphase FISH analyses. Second-line therapy was started CAB in all patients [Flutamide forty-four patients, bicalutamide twenty-nine patients and CPA for four]. Clinical outcome was monitored mainly by PSA decline/increase every three months, and clinical symptoms.

2. Techniques of molecular cytogenetics

2.1. FISH analysis

The fluorescence in situ hybridization [FISH] analysis of the AR gene copy number was performed as previously described (Hyytinen et al., 1994; Visakorpi et al., 1995). Briefly, a genomic P1-probe for AR gene [LCG-P1AR] was labelled with biotin-16-dUTP [Roche Ltd, Mannheim, Germany] using nick-translation. A Texas-Red-dUTP [DuPont, Boston, MA] labelled chromosome X α-satellite [DXZ1] probe was used as a reference in hybridization. The interphase nuclei from the paraffin-embedded tissue sections were disaggregated and spotted on slides. The samples were then

pretreated with glycerol solution at +90 °C followed by proteinase K treatment, then denatured and hybridized. After hybridization, the slides were washed and the AR probe visualized with avidin-fluorescein isothiocyanate [Vector Laboratories, Burlingame, CA]. The AR copy number was scored independently by two observers [CP and LK] from about one hundred nuclei per cell using an Olympus BX50 epifluorescence microscope [Tokyo, Japan]. Scoring was done without knowledge of the clinical course of the patients. The interpretation of results followed the guidelines described elsewhere. The criteria for amplification included the presence of individual tumour cells with tight clusters of AR signals, with more than 5 signals per cell or with a more than two-fold higher number of AR than reference [DXZ1] signals. A minimum of 70 nuclei was counted pro slide.

2.2. Immunohistochemistry [IHC] and in situ end-labelling [ISEL]

The proliferation activity of tumours was determined by Ki-67 IHC using standard immunoperoxidase staining. A novel mouse monoclonal Ki-67 antibody [clone MM-1, Novocastra Laboratories, Newcastle, UK] was used at a dilution of 1:1000 after high temperature antigen unmasking [10 minutes boiling in a 0.01 mol/l citrate in buffer in an autoclave at 120°C, pressure 1.05 bar]. The bound antibody was visualized using the standard avidin-biotin technique [Vectastain Elite, Vector Laboratories, Burlingame CA]. The apoptosis was measured by in situ end-labelling technique using the ApopTaq-kit [Oncor Inc., Gaithersburg, Maryland, USA] according to manufacturer's instructions. Prostates from castrated and non-castrated rats were used as control in apoptosis staining.

For immunohistochemical staining of p53 protein, three-micrometre thick sections were cut from paraffin-embedded blocks on ChemMate^{TM} capillary gap microscope slides [Dako a/s, Glostrup, Denmark]. Before immunostaining rehydrated sections were heated in a microwave oven at 850 W for two 7-minute cycles using 0.01 mol/l citrate buffer [pH 6.0] as antigen retrieval solution. Staining was performed using the indirect streptavidin-biotin peroxidase method in a TechMate^{TM} 500 Immunostainer [Dako a/s]. Primary mouse antibody to human p53 [clone DO7, Novocastra Laboratories Ltd, Newcastle, UK], diluted 1:40, was visualized with a ChemMate^{TM} detection kit [Dako a/s] with diaminobenzidine as chromogen. Staining results were scored in blinded fashion [i.e. unaware of pairing and identity of samples] and expressed according as p53 staining was present or not. Specimens with intense

nuclear immunoreactivity in more than 10 % of the malignant cells were regarded as p53-positive.

Labelling index

The apoptotic index [AI] was defined as the percentage of ISEL-positive cells relative to counted carcinoma cells. The AI was determined by counting 150 malignant cells in 20 fields [4-8 fields in TruCut samples] at x 400 magnification. The proliferative index [PI] was obtained as the percentage of Ki-67 -immunopositive nuclei and determined in the same manner as described for the AI.

2.3 Mutation analyses

Twelve cases with AR amplification [study III] were studied for the presence of mutations in the amplified AR gene using the standard single stranded conformation polymorphism technique [SSCP].

2.4 Transfections

To study the transactivating function of a mutated AR gene [study III], the mutation was introduced into the AR expression vector $pSVAR_0$. The functionality of the mutated AR was analyzed in Hep3B cells in the absence or presence of synthetic androgen R1881 or antiandrogen flutamide.

3. Statistical methods

Statistical analyses were performed using the GraphPad InStat and BMDP Statistical Software Package [Dixon WJ. BMDP Statistical Software. Berkeley, Los Angeles. University of California Press: London, 1981.]. The clinopathological parameters were compared using Pearson chi-square test, Fisher's exact test [two-tailed] and variance analysis. The statistical significance of survival differences between patient groups was determined by Mantel-Cox test and Breslow tests [BMDPIL]. In addition, in Study I Cox uni- and multivariate analyses (Cox, 1972) were used to calculate the relative risk ratios [RR] and their 95 % confidence intervals [95 % CI] and to test the independence of prognostic factors. The analyses were made using cancer-specific survival rates. In Study II correlation between apoptotic index and proliferative index was calculated using Spearman rank correlation, and comparison of survival curves was by log-rank test.

4. Ethical aspects

The ethics committee of Tampere University Hospital accepted the protocols. The patients in the prospective trials were recruited in accordance with the Declaration of Helsinki.

RESULTS

1. Androgen sensitivity of primary prostate cancers and PSA as a prognostic marker (Study I)

In only 14 out of 236 [6%] patients PSA declined less than 50% from the value at the time of diagnosis, which suggests that only a few primary CaPs are androgen-insensitive at the primary stage. PSA decline after primary hormonal therapy was a significant and independent prognostic marker for CaP-specific survival [p<0.001, Figure 1, article I, page 193]. In multivariate analysis, only M stage, histological grade and the PSA-based response class remained as independent prognostic markers.

2. Primary androgen-insensitive prostate cancer (Study II)

The proliferation index measured by Ki-67 was only slightly higher [p=0.27] in the primary androgen-insensitive group. There was no correlation between the grade and the stage of the disease. The apoptotic index was almost three times higher in the androgen-sensitive group [p=0.0001]. The result did not correlate with the grade or stage of the disease. Expression of p53 was found in two [10%] in the androgen-sensitive and in three [30%] of the androgen-insensitive group (p=0.30). Proliferative and apoptotic indices showed no significant correlation within the groups. Prostate cancer-specific survival: in the androgen-sensitive control group the median survival was 38.1 months [range 12.5-141.0] and as against in the androgen-insensitive group 11.9 months [range 5.3-17.4; p=0.0001].

3. Biological and clinical characteristics of prostate carcinomas containing AR gene amplification (Study III)

Fifteen [28%] of the 54 recurrent therapy-resistant tumours, but none of the untreated primary tumours, contained AR gene amplification as determined by interphase FISH analysis. The mean AR copy number per cell ranged from 2.7 to 28, with variation between the cells to a maximum copy number of 60 copies per cell. Thirty-nine out of 54 tumours [72%] had only one copy of the AR gene and the reference probe DXZ1.

The AR gene amplification was not significantly associated with the age of the patient or with the stage or grade of the tumour. A significant association was detected with both the degree and the duration of response to primary hormonal treatment [Figure 1 A and B, article III, page 316]. Patients with AR amplification all evinced partial or

complete response to the primary therapy and the duration of the response was at least one year. Both overall and median post-recurrence survival were also found to be significantly longer in the amplified group [Figure 2 A and B, article III, page 317].

mRNA in situ hybridization was performed in six cases and showed high expression of mRNA in amplified tumours, suggesting that the gene was active. One point mutation with alteration in the structure of the codon was detected in an amplified tumour, but this seemed to have no influence on the function of the receptor gene.

4. AR gene amplification after monotherapy with nonsteroidal antiandrogen (Study IV)

High-level amplification was found in the recurrent CaP specimens with up to thirteen AR gene copies per cell nucleus in a patient receiving antiandrogen monotherapy as primary treatment. No amplification was found in the primary tumour. Third-line CAB by adding LHRH analogue to bicalutamide after cytotoxic therapy evoked an excellent but short-lived response with both PSA decline and normalized PSPA-score.

5. AR gene amplification as a predictor of response to combined androgen blockade (Study V)

In the whole material [n=77] AR gene amplification was found in ten [13%] tumours at the time of progression. In a more selective group of patients with signs of local recurrence of the CaP, amplification was detected in seven [18%] patients. Of the nine patients treated by TURP for local tumour progression, amplification was observed in three [33%] tumours.

AR gene amplification was not associated with the grade or the stage of the disease, neither with the age of the patient at the time of diagnosis or the pre-treatment PSA level. There was no association with the PSA level at the time of relapse and the amplification of the AR gene or with the time elapsing from initiation of the primary treatment to the time of relapse. AR gene amplification was significantly associated with the PSA-based response rate to second-line CAB [p=0.016; Figure 1 A, article V, page 3] in the whole material, and even in the subgroup of patients experiencing local recurrence [p=0.017; Figure 1 B, article V, page 3]. It was also seen that

patients with AR gene amplification showed more often a decreasing PSA level after the introduction of CAB than patients without the amplification, but there was no significant difference between the groups [p=0.079 for the whole group, p=0.073 for patients with local recurrence] [Table 4, article V, page 3]. There was no benefit in terms of survival for patients with AR gene amplification.

DISCUSSION

1. Androgen sensitivity of primary prostate cancer

In previous studies it has been assumed that 60-90% of the newly diagnosed CaPs are androgen-sensitive and show a positive response to hormonal therapy (Grayhack et al., 1987; Mahler and Denis, 1995). Thus, 10-40% of patients should not respond and should relapse shortly after commencement of endocrine treatment. These previous studies have mainly used National Prostate Cancer Project [NPCP] criteria (Murphy and Slack, 1980) for definition of treatment response. In contrast, the current study (I) was carried out to evaluate the frequency of primary androgen-independent, or insensitive, prostate carcinomas as reflected in serum PSA decline.

The synthesis and production of PSA is strongly dependent on androgens and therefore the decline in PSA after the initiation of hormonal therapy may be attributable to the diminishing of cancerous tissue, and/or the suppression of the strongest stimulator of its expression, the androgens. Thus, PSA decline after androgen ablation probably indicates both a decreasing tumour burden and a direct response to decreased levels of androgens in the serum. Nonetheless, the PSA level usually increases at the time of CaP progression even during androgen deprivation (Fowler et al., 1995).

In our material, only 14 patients out of 236 [6%] showed less than 50% decline or increase in PSA levels during the first year from commencement of androgen ablation, which would imply that almost all CaPs are androgen-responsive at the time of diagnosis. Of these fourteen patients, two had increased PSA levels compared with baseline at three months and two at six months and four at twelve months; thus the majority of these non-responders still had a short-term decline in their PSA level. Similar biochemical response rates have been reported elsewhere (Smith et al., 1997). It would seem that the estimation of the response rate for androgen ablation is highly dependent on the parameter used to define the response [NPCP criterion or PSA levels].

2. PSA as a prognostic marker in hormonally treated prostate cancer

In view of the strong association between PSA increase and progression of disease, PSA has been widely used in the follow-up of CaP. The increase in PSA levels seems to precede a clinical relapse by months, and it has therefore also been used as a surrogate end-point in clinical trials (Kupelian et al., 1996; Newling, 1993). In this study (I), we assessed the prognostic value of PSA response in hormonally treated CaP. Univariate analysis showed PSA decline to be strongly associated with survival. The nonresponders had extremely poor prognosis, with a median survival just over one year. The fact that non-responders had clearly poorer prospects than the patient in the stable disease group suggests that our initial, more or less arbitrary, categories of treatment responses do in fact well characterize the androgen responsiveness of the tumours. In multivariate analysis, the PSA response showed independent prognostic value together with M stage and histological grade. In the analysis, the PSA response entered the model first, suggesting that the PSA decline reflects the impact of other prognostic markers.

It has also been suggested that the pretreatment PSA level is associated with disease progression (Lerner et al., 1996), and we indeed found the pretreatment value to be a strong prognostic factor. On the other hand, multivariate analysis indicated that it had no independent prognostic value. Pretreatment ALP values have been held to correlate with prognosis (Reynard et al., 1995; Smith et al., 1997) and the ALP was associated with PSA response in this study. Since this value had been measured in only 64 % of the patients, however, it was not included in the survival analysis.

3. Identification of patients with primary androgen-insensitive disease

According to study I, six per cent of CaP patients do not respond to conservative hormonal therapy with androgen deprivation. Their cancer-specific survival is significantly inferior to that of patients evincing some response to hormonal treatment. Cytotoxic therapy has been shown to have only limited efficacy as a second-line therapy for hormone-refractory CaP (Fosså and Paus, 1994). Recently there have been some suggestions that cytotoxic therapy as primary treatment alone or in combination with hormonal therapy might be effective (Eilon et al., 2000; Ferrante et al., 1999; Millikan, 1999). It would be therefore important to be able to identify this patient category at the earliest possible stage of disease. The aim of

Study II was to find histopathological parameters which could be used to identify such primary androgen- insensitive tumours.

Proliferative activity has been shown to be an independent prognostic marker for patients who have had radical prostatectomy (Bettencourt et al., 1996; Bubendorf et al., 1996) and also for hormonally treated patients (Ahlgren et al., 1999; Baretton et al., 1999; Visakorpi et al., 1991). In the present study, proliferative activity did not differ between the primary androgen-insensitive and androgen-sensitive cancers. Another well known prognostic marker in CaP is p53 (Visakorpi et al., 1992b), which also failed to differentiate androgen-insensitive and dependent tumours. On the other hand, the primary androgen-insensitive tumours showed a three-fold lower apoptotic activity than hormone-sensitive tumours. Thus, measurement of apoptotic activity might be useful in detecting primary androgen-insensitive disease. Larger studies to evaluate the apoptotic index as predicting the presence of hormone-independent CaP are thus warranted.

4. Androgen receptor and emergence of hormone-refractory prostate cancer

Although the majority of CaP patients show clear response to hormonal treatment, the disease will eventually always progress. The emergence of hormone-refractory CaP is one of the most serious clinical problems in the management of the disease. The mechanisms underlying the progression are inadequately known. In 1995, Visakorpi and colleagues (1995) reported that AR is amplified in a subset of hormone-refractory CaP. This suggested a new mechanism to explain and understand therapy failure in CaP during conventional hormonal therapy. In the present study, AR gene amplification was found in 28% of hormone-refractory CaPs. It was associated with increased levels of mRNA expression of the gene, as expected from the true target gene of amplification. Amplification was not found in tumour samples taken prior to hormonal therapy, suggesting that the castration had selected the tumour clone containing the amplification. In the majority of cases the amplified AR gene was a wild-type gene, further indicating that the amplified AR gene is functional. The patients with AR amplification evinced a longer and more complete response to primary hormonal therapy than those without amplification, which would indicate that tumours which acquire the amplification are already highly

hormone-sensitive at the beginning of the disease. The patients with the AR amplification also had better survival than those without, further indicating the androgen sensitivity of these tumours.

Altogether, the data suggested that failure of conventional androgen deprivation therapy might be caused, at least partly, by a clonal expansion of cancer able to utilize the AR signalling pathway even in an environment with only a low concentration of androgens. The relapsed cancer may in fact not be truly androgen-independent but hypersensitive.

5. Detection of androgen receptor gene amplification as a predictive marker

The significance of adrenal androgens remaining in the serum after castration is poorly known. Although some studies have reported that combination of antiandrogen with castration improves the response rate (Crawford et al., 1989; Labrie et al., 1986; Schellhammer, 1996), subsequent studies have not confirmed this (Eisenberger et al., 1998; Prostate Cancer Trialists' Collaborative Group, 1995). It has also been suggested that antiandrogens could be beneficial as a second-line therapy after conventional androgen deprivation has failed. Up to 20-30 % of patients have been reported to respond to CAB as a second-line therapy (Geller and Albert, 1985; Labrie et al., 1988), but usually only for short periods.

In the present study (IV and V) the use of AR copy number analyses as a predictive marker for combined androgen blockade was investigated. First, we demonstrated amplification of the AR gene during nonsteroidal antiandrogen monotherapy. The patient in question also had a good response to third-line CAB. Based on the finding we suggested that patients with amplified AR gene might benefit from CAB as second-line therapy.

In a prospective study (V), we investigated the value of AR gene amplification in predicting the response to second-line CAB treatment. We found that patients whose tumours contained the gene amplification at the time of the initial progression responded significantly better to CAB treatment than those without amplification. Thus, the detection of AR gene amplification might prove a clinically useful biomarker, whose presence predicts favourable response to second-line CAB

therapy. It should be noted that this assay would not be useful in predicting response to CAB as first-line endocrine therapy, since AR gene amplification is not found in untreated prostate carcinomas.

The detection of AR gene copy number in the study was based on prostate biopsies taken at the time of primary progression. Only 13% of cases showed AR amplification. This is clearly less than we found in locally recurrent hormone-refractory tumours from patients treated by transurethral resection [TURP] for urethral obstruction (III) or what has been reported in hormone-refractory metastatic lesions (Bubendorf et al., 1999). It is most likely that in many cases the local tumour and progressing metastatic lesions represent two different tumour clones. Thus, the prostate biopsy taken at the time of progression may not represent the progressing tumour. In addition, intraprostatic tumour heterogeneity may strongly influence analysis especially when needle biopsies are used. When we restricted our analyses to patients who experienced local progression, 17.5% of cases showed AR gene amplification, whereas 33 % of those who underwent TURP because of severe urethral obstruction showed amplification. The latter frequency is in good agreement with that observed in our previous retrospective study (III) and demonstrates the sample biases in our prospective study.

In order the better to utilize biomarkers in predicting response to second-line treatment new tools for sample collection should be developed. Since most patients experience progression in the form of distant metastases, samples from the metastases should ideally be analysed. One possibility in this respect is to obtain bone marrow [BM] samples. More than half of the patients with early recurrence after radical prostatectomy show micrometastatic cells in the bone marrow samples taken before the operation (Gao et al., 1999). The same is true for CaP patients with hormonally treated advanced or metastasized disease (Wood et al., 1994).

Although patients with AR amplification responded better to CAB than those without, there was no survival benefit for the former. From the clinical trials it is evident that current antiandrogen therapies are not effective enough to improve survival after the relapse of primary hormonal treatment, this not even in cases we assume to be androgen-hypersensitive due to AR gene amplification. Thus, it will be important in the future to develop new treatment modalities, which block the stimulatory effects of the AR signalling pathway.

SUMMARY AND CONCLUSIONS

Advanced prostate cancer cannot be cured. However, vast majority of prostate cancers is androgen-sensitive in the primary stage and can be effectively treated with androgen manipulative therapy for variable period of time. A small subset (6%) of prostate cancers seem, however, to be primarily androgen-insensitive. It appeared, moreover, that establishment of apoptotic index may provide a tool to identify such tumours. In addition, PSA decline during the first year was shown to be an independent prognostic marker for patients treated with hormonal therapy. The most critical problem arises when primary hormonal therapy fails and a hormone-refractory tumour emerges. Combined androgen blockade is a treatment modality to block both testicular and adrenal androgens, and as a second-line therapy it gives benefit to about 30% of patients. This study showed that detection of AR gene amplification could be used to predict the response to second-line CAB and thus to identify those patients who might benefit from this form of therapy.

As a conclusion, this thesis suggests, that

1. since only 6 % of the patients with advanced prostate cancer showed no response to hormonal treatment measured by the decline in PSA, it seems that primary CaP is more sensitive to hormonal treatment than previously assumed.
2. PSA decline is useful marker in predicting disease outcome after hormonal therapy.
3. primary hormone-insensitive or independent CaP shows significantly lower apoptotic activity than hormone-sensitive tumours.
4. nearly one third of locally recurrent CaPs contains AR gene amplification. These tumours have initially responded well to endocrine therapy and their response have lasted longer than average. The median survival time after recurrence is twice as long for patients with AR amplification in comparison to those with no amplification.
5. AR gene amplification detected in tumours progressing during androgen deprivation therapy or antiandrogen monotherapy is associated with favourable response to second-line combined androgen blockade. This, on the other hand, suggests that at least some of AR amplified tumours retain a high degree of dependency on the residual androgens in serum after primary hormonal therapy and that detection of AR gene copy number may be a useful predictive marker.

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