

**ANDROGEN RECEPTOR GENE AMPLIFICATION IN
BONE METASTASES FROM HORMOME-
REFRACTORY PROSTATE CANCER**

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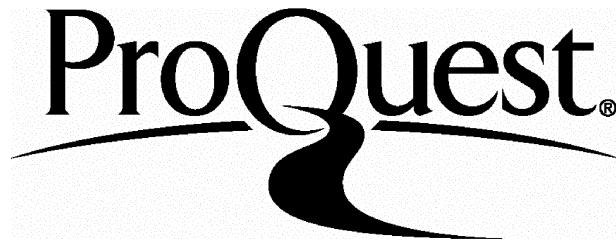
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Abstract

The mechanisms for hormone-refractory prostate cancer (HRPC) are incompletely understood. There is no effective therapy for HRPC and new treatment approaches are needed. One postulated mechanism for the development of HRPC is androgen receptor (AR) gene amplification in prostate cancers treated with androgen deprivation or anti-androgen therapy. By increasing the availability of nuclear androgen receptors, cells with AR gene amplification may continue to more efficiently utilise low levels of circulating androgen found after castration. The AR gene amplification may therefore confer a growth advantage for tumour cells showing these changes, compared to cells without such a mutation.

Androgen receptor gene amplification has been demonstrated to occur in approximately one-third of patients with either local tumour recurrences from HRPC or at the sites of metastatic disease studied to date. Samples from bone metastases in patients with HRPC are difficult to both obtain and work with and androgen receptor gene amplification has not been studied or demonstrated in bone metastases from HRPC.

This thesis describes a method for obtaining bone metastases from HRPC patients using bone marrow trephine biopsies, targeted by diagnostic bone scans. The prevalence of AR gene amplification in the metastases obtained is examined. Data on DNA preservation for fluorescent *in situ* hybridisation (FISH) studies to demonstrate AR gene amplification following acid decalcification of bone samples is presented. Unsuccessful attempts at studying the HRPC samples using comparative genomic

hybridization (CGH) are detailed. Androgen receptor immunohistochemical (IHC) expression in these bone metastases is also assessed to evaluate if IHC can identify tumours exhibiting AR gene amplification.

Patients with AR gene amplification at primary tumour progression may respond more favourably to subsequent combined androgen blockade. Demonstrating AR gene amplification at a clinically important disease site, may allow sub-classification of HRPC patients who can benefit from this or alternative therapeutic strategies targeted at AR gene amplification.

Dedication

To Patricia, Anna and Oliver for their love and support.

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Chapter 1:

Introduction

1.1 Introduction

Androgens are the principal male sex hormones, with a wide range of physiological actions and functions. The castration of domestic animals (and humans!), performed in many societies for thousands of years, suggests that some level of understanding of normal testicular function in the role of maintaining ‘maleness’ has been appreciated for a long time. The Muslim empires from 750 AD and the ancient Chinese, both advocated the use of testicular extracts as aphrodisiacs and for the treatment of impotence and hypogonadism (Belchetz 1998). Chinese alchemists, between the 10th and 16th century developed remarkably advanced techniques for extracting near-pure hormones from urine. Diseases associated with defects of these hormones were recognised relatively early in medical history, but it is only in the last 100 years that there has been a significant increase in our knowledge of their role in physiology and disease.

As part of the same family of steroid hormones as the female sex hormones, glucocorticoids, mineralocorticoids and vitamin D₃, androgens are derived from the cholesterol molecule. In men, approximately 90-95 % of circulating androgens are produced in the testis, with the adrenal gland as a secondary site of androgen production.

Testosterone, the principle androgen, and is produced by the Leydig cells of the testis. Production is under the control of luteinising hormone (LH), produced in the anterior pituitary. This in turn is controlled by luteinising hormone-releasing hormone (LHRH) from the hypothalamus. A negative feedback loop control

mechanism is completed by the action of testosterone on the hypothalamus. Testosterone enters the target cell by diffusion and either associates directly with the AR or is metabolised to 5-alpha-dihydrotestosterone (dihydrotestosterone), a higher affinity ligand for androgen receptor (AR) and the most active naturally occurring androgen (McPhaul *et al.* 1993). Dihydrotestosterone is produced by the enzymatic action of 5-alpha-reductase on testosterone. Two isoforms of 5-alpha-reductase are known, with the type II isoenzyme believed to be the most important in prostatic physiology. Dihydrotestosterone appears to be the most important androgen in prostatic development (Wilson 1996) and has almost seven times greater affinity for AR than testosterone.

The adrenal gland is the second site of androgen synthesis. In this pathway, corticotrophin-releasing hormone stimulates the release of adrenocorticotrophic releasing-hormone from the pituitary. This in turn leads to the production of the adrenal androgens androstenedione and dihydroepiandrosterone. These adrenal androgens enter the circulation and dihydroepiandrosterone is converted to testosterone and dihydrotestosterone both within plasma and in 'end-organs', such as the prostate, which can directly metabolise adrenal androgens.

1.2 ANDROGENS AND THE HUMAN PROSTATE

1.2.1 The Role Of Androgens In Embryonic Human Prostate Development

All male mammals have one or more accessory reproductive glands. In man, one of these, the prostate, lies at the neck of the bladder with the urethra traversing through it. In humans, at approximately 8 weeks of gestation, Leydig cells of the developing testis have begun to produce androgens (Siiteri *et al.* 1974). Soon after, by 10 weeks gestation, an early prostate gland is recognisable, developing from outgrowths of the urogenital sinus. Ablation of the foetal testis during this ambisexual period of sex differentiation completely inhibits development of male internal sex glands including the prostate (Cunha *et al.* 1987). By the end stages of foetal growth, early primitive, solid, prostatic epithelial buds have formed a complex tubuloalveolar gland, pyramidal in shape, with the base at the bladder neck and the apex on the urogenital diaphragm. In rodent models, this budding prostatic glandular epithelium appears to result from the action of 5-alpha-dihydrotestosterone on androgen receptors, which can be identified early on within the mesenchymal tissue (Shannon *et al.* 1993). Testosterone appears to have the major role in embryonic virilization of the Wolffian duct in the male, but the androgenic action on the urogenital sinus (from which the prostate develops during embryogenesis) is mediated largely or exclusively by the dihydrotestosterone-AR complex (Wilson 1996).

A rare disorder, 5 alpha-reductase type-2 deficiency, has been described in certain pedigrees in the Dominican Republic, New Guinea and Turkey and results in reduced, but not absent, dihydrotestosterone production (Wilson *et al.* 1993). This

disorder is associated with impaired development of dihydrotestosterone-dependent end organs such as the prostate, beard growth and male pattern body hair. In males, the prostate is usually present but rudimentary in nature. Males have normal testes and internal genitourinary ducts but ambiguous or female genitalia at birth. At puberty, the testes descend into the labioscrotal fold, which becomes pigmented and rugated. Partial virilization also occurs, but the prostate remains small, approximately one-tenth the size of age-matched controls (Imperato-McGinley *et al.* 1992). Psychosexual orientation may be problematic. Biochemically, all affected individuals show deficiency in DHT production, with a testosterone: DHT ratio approximately 3-5 times higher than normal and a mean serum DHT level approximately one third of normal.

A further crucial series of observations on basic prostate embryology and organogenesis has been provided by studies on the testicular feminization-mouse (tfm) model. The tfm mouse is insensitive to androgenic action. Androgen receptors are undetectable and cannot interact in the nucleus with chromosomal DNA. Genetically male, tfm mice are phenotypically female, with the development of a vagina and no prostate. The recognition of this type of mutation documented the critical role of AR in the normal embryonic action of androgen and prostate development (Wilson *et al.* 1981).

In a further series of classic co-culture experiments to determine the nature of the stromal-epithelial interaction in the developing prostate, Cunha and colleagues showed that embryonic wild-type prostatic stromal cells from the urogenital sinus mesenchyme when co-cultured with embryonic tfm-epithelial cells (normal apart

from the ability to respond to androgenic stimulation) developed into an explant prostate that contained normal prostatic acini and secretory products (Cunha *et al.* 1981). When the reverse experiment was done (i.e. androgen impaired embryonic tfm-stroma cells were co-cultured with wild-type embryonic prostatic epithelium), no prostate acini and secretory products developed. These findings have provided some of the strongest evidence to date to suggest that stromal cells direct the development of prostatic epithelial acini embryonically, and that the reverse is not true.

1.2.2 The Role Of Androgens In Pubertal Prostate Development.

Puberty represents a second major stage of prostatic glandular development after the acinotubular activity of the late foetal period (Cunha *et al.* 1987). At puberty, the human prostate gland develops adult characteristics and function. During this period, prostatic growth results from androgen-dependent cell proliferation that exceeds cell death rates (Cunha *et al.* 1987). The normal human adult prostatic size is not achieved until approximately 20 years of age (Janulis *et al.* 2000), several years after the initiation of puberty.

1.2.3 The Role Of Androgens In Maintaining The Human Adult Prostate.

One of the few recognised functions of the prostate is as an exocrine gland (Janulis *et al.*, 2000). Prostatic epithelial secretions released into the acinus, are carried through the duct system and empty into the urethra. Here these contents are mixed with

seminal fluid, and are believed to be important in the maintaining and transporting of sperm. Many different molecules are secreted into prostatic fluid, and their exact roles and character is incompletely understood. Some, such as PSA, a serine protease, are believed to aid semen liquefaction. Other molecules such as citrate, fructose, selenium, zinc, and polyamines have a less obvious role (Arnold *et al.* 2002).

In the presence of physiological androgens, the normal adult prostate is in a steady and self-renewing state. The epithelial compartment is composed of a mixture of androgen-dependent (e.g. terminally differentiated luminal secretory cells), androgen-sensitive (e.g. transient amplifying epithelial cells) and androgen independent epithelial cells (e.g. basal epithelial stem cells, Arnold *et al.* 2002). The glands are surrounded by a basement membrane, separating them from the fibromuscular stroma. This physically supports the glandular epithelium and contributes to the endocrine and paracrine microenvironment (Arnold *et al.* 2002).

That the adult animal prostate function remains androgen-dependent is perhaps most obviously demonstrated after the sudden removal of testicular androgens by castration. This results in a rapid and significant reduction in the size of the gland, and has been recognised for over 100 years (Cunha *et al.* 1987). On this basis, castration has been a treatment of human prostatic conditions such as benign prostatic hypertrophy (White 1895; Huggins *et al.* 1940) and prostatic carcinoma (Huggins *et al.* 1941). Detailed studies of the early and late effects of castration have been studied in rat, mouse and dog prostatic animal models rather than in humans (Sinowatz *et al.* 1995). In patients undergoing male-female gender reversal, long-

term androgen deprivation results in atrophic changes in the prostate, which appear not to affect prostatic size but results in histological atrophic changes (de Voogt *et al.* 1987).

In the rat, within one or two days post castration, there is little or no change in weight, nucleic acid or protein content of the prostate. This is followed by a period of rapid loss of fresh weight, DNA, RNA and protein such that after a week, there is less than 15% of the original weight, RNA and protein content of the prostate remaining (Lee 1996). These striking and rapid changes are associated with major histological alterations in the epithelial acini, the result of massive epithelial apoptosis (Lee 1997). Apoptotic death appears most marked at the distal and intermediate regions of the acinus while cells of the proximal duct can survive androgen deprivation. Changes in the stromal morphology also occur despite little change in stromal mass, at a much slower rate. The net result appears to be an increase in androgen-independent cells that can survive at castrate levels of circulating androgens as long as the animal lives (Lee 1997).

Androgen receptor location in the normal adult human prostate has been studied by a number of groups (Bashirelahi *et al.* 1979; Ruizeveld de Winter *et al.* 1991; Brolin *et al.* 1992; Chodak *et al.* 1992; Bonkhoff *et al.* 1993; Kimura *et al.* 1993). As part of a body-wide survey of androgen receptor immunohistochemistry, Ruizeveld de Winter *et al.* were one of the first groups to apply this technique to the prostate. They showed exclusive nuclear staining of columnar epithelial cells of the prostate without basal epithelial staining in 2 patients. Stromal cells of the prostate exhibited variable staining patterns. Chodak in 1992 confirmed these findings. In contrast, Bonkhoff

was able to show widespread nuclear androgen receptor staining in basal cells using a double staining technique for basal cell-specific cytokeratins. In comparison to secretory luminal cells, AR was more frequently expressed in smaller quantities on basal cells. Focal areas of strong basal cell staining were however seen. Smooth muscle cells of the prostatic stroma were found to express AR by Kimura (*Kimura et al.* 1993). Overall, there remains conflicting evidence as to whether adult prostate basal cells express AR.

1.2.4 Androgens, Senescence And The Prostate.

The human prostate shows progressive, androgen-mediated growth during the later years of life (*Prins et al.* 1996), on the background of a period of relative quiescence in size during early and mid-adulthood (*Janulis et al.* 2000). In contrast to this, male human serum testosterone levels decline in a more variable way with age, unlike the female menopause in which oestrogen levels drop significantly. Plasma levels of testosterone below the normal range have been documented in 35 % of men over 80 and in 20 % of men aged 60-80 (*Belchetz 1998*). The evaluation of the role of androgen activity in the ageing male prostate is severely hindered by the absence of a defined distinction between what is normal and what is disease (*Montie et al. 1994*).

Associated with increasing age, and the slow decline in serum testosterone levels, is the development of hyperplastic change known as benign prostatic hypertrophy (BPH) and the development of associated urinary outflow symptoms. Almost the

only other species apart from man known to develop BPH is the domestic dog (Wilson 1980).

The pathogenesis of BPH is not fully understood. Four hypotheses have been formulated with dihydrotestosterone, oestrogens, stromal-epithelial interactions and stem cell changes all having been implicated (Srinivasan *et al.* 1995). The prevailing current view with regard to androgen levels within prostatic BPH is that testosterone and dihydrotestosterone do not appear to be elevated in comparison with the normal prostate (Montie *et al.* 1994). BPH is however, known to develop only in the presence of intact testes, and androgens are therefore felt to play an important role in BPH. Prepubertal castration prevents the occurrence of BPH (Scott 1953).

The effectiveness of 5- α -reductase type II inhibitors in the treatment of BPH is evidence that dihydrotestosterone may still be still important despite the age-related decline in circulating androgen levels. BPH is also believed to be associated with a state of relative dihydrotestosterone excess as testosterone levels decline (Wilson 1996). The relationship between plasma and tissues levels of testosterone and dihydrotestosterone is also not yet clearly defined, hampering the understanding of the role of sex hormones in the pathogenesis of BPH (Montie *et al.* 1994).

1.3 THE ANDROGEN RECEPTOR

1.3.1 The Hormone Receptor Superfamily.

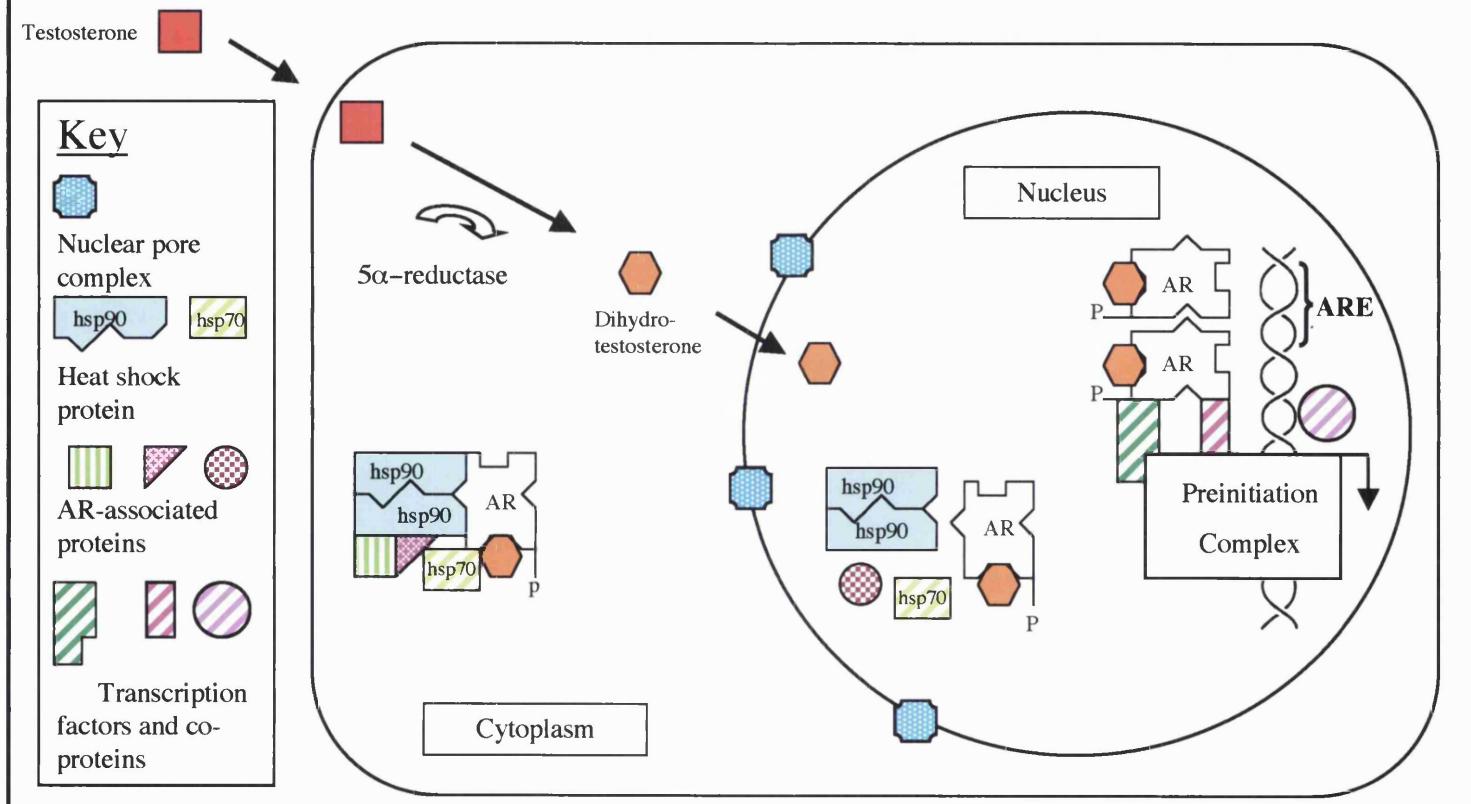
The androgen receptor is one member of the hormone receptor, ligand-dependent, transcription factor superfamily that act by binding to DNA, inducing gene transcription. The family includes the steroid hormone receptors; AR, oestrogen α and β receptors (ERs), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) as well as other hormone receptors such as the thyroid hormone receptors α and β (TRs), the vitamin D receptor (VDR) and several types of retinoic acid receptors (RXRs). Other structurally similar hormone receptors have been identified without specific ligands recognized for them. They have been termed orphan receptors.

1.3.2 The Normal Androgen Pathway.

Before considering specific details of the AR and its interactions, it is necessary to briefly outline the current model of prostatic androgen action (see **Figure 1.1**). The sequence starts with the passive diffusion of free testosterone into target cells within prostatic tissue. Testosterone is then converted in the cytosol to dihydrotestosterone by the enzyme 5-alpha-reductase type II. The subcellular localisation of the AR in either the presence or absence of ligand is still a point of discussion. Ligand-receptor binding has been reported to occur both within the cytosol (Jenster *et al.* 1991; Simental *et al.* 1991; Jenster *et al.* 1993), and more recently it has been thought that

unliganded and ligand-AR complexes are primarily located within the nucleus (Prins 2000).

Figure 1.1. Intracellular actions of androgens within prostate cells



Regardless of subcellular localisation, androgen receptors are believed to be associated with a number of heat shock proteins (HSPs) particularly HSP 56, 70 and 90 in the form of a heteromeric complex (Smith *et al.* 1993). HSPs are thought to stop unliganded action of the receptor. The binding of androgen to AR coincides with the dissociation of HSPs and conformational activation of the hormone-receptor complex (Pratt 1993). The conformational change is related to hormone-dependent (but DNA-independent) phosphorylation of the receptor at several sites (Prins 2000) and is believed to be an essential process for AR activation (Grossmann *et al.* 2001). This phosphorylation is additional to a hormone-independent phosphorylation that occurs soon after production of the receptor (resulting in an increase in molecular weight from 110 to 112 kilo-Daltons (kDa) in human LNCaP cells in the absence of androgen). Both the 110 and 112-kDa isoforms can undergo ligand-dependent transformation that leads to nuclear binding (Kuiper *et al.* 1991).

Within the nucleus, homodimerisation of two AR proteins occurs and is essential for hormone activity (Truss *et al.* 1993). Homodimers of the ligand-receptor complex binds to specific Androgen-response elements (ARE's) of chromosomal DNA in target genes by means of two zinc finger structures within the DNA binding domain (made available by androgen-induced conformal changes in the receptor). Binding of AR dimers to DNA is associated with hyperphosphorylation, mediated through the action of hormone-dependent, DNA-dependent phosphokinase (Prins 2000). The results of this activated receptor-DNA binding is the association of the 'hyperphosphorylated' steroid receptor-DNA complex with the preinitiation transcription complexes of the promoter region of the hormone-regulated gene. Accessory factors are required for efficient nuclear transcription and act as signalling

intermediaries between the receptors and the general transcription machinery (McKenna *et al.* 1999). Some of these factors (termed co-activators or co-repressors) are thought to be able to interact with most steroid receptors while others are suspected to be steroid receptor-specific. The result of transcription of AR-regulated genes is the appearance of new nuclear (and subsequently cytoplasmic) mRNA that acts as the template for ribosomal protein production within the cell by translation. Androgen-dependent proteins are thereby produced, capable of carrying out androgen-dependent prostatic functions.

The Normal Androgen Receptor

The androgen receptor was first described in 1969 in the rat ventral prostate (Fang *et al.* 1969). To date, only a single human androgen receptor has been identified, and the exact mechanisms for the differing effects of testosterone and dihydrotestosterone on this receptor are not fully understood (the first report of a androgen receptor subtype in any species, was reported in 1999 from a Japanese species of eel, Ikeuchi *et al.* 1999).

The location of the human androgen receptor gene was confirmed as the Xq11-12 position (proximal long arm) of the X chromosome by Kuiper *et al.* and Brown *et al.* in 1989 (Brown *et al.* 1989; Kuiper *et al.* 1989). This followed the recognition during the 1970s (Meyer *et al.* 1975), that human androgen insensitivity syndromes were inherited as X-linked traits. The single AR gene on the X chromosome spans approximately 90 kilo-bases (kB) of genomic DNA and contains 8 exons. Human

AR cDNA is 10.6 kB in size; 1.1 kB of 5'-untranslated region (UTR), an open reading frame (ORF) of 2.7 KB and 6.8 kB of 3'-UTR (Faber *et al.* 1989). Exon 1 is the largest exon and encodes the N-terminus transcription activation domain. The second and third exons encode the two DNA binding domains of the protein. The remaining five exons code for the androgen-binding domain (exons 4-8) (Taplin *et al.* 2001).

Perhaps the most significant advance in the study of the human androgen receptor to date came in the late 1980s when several groups published the cDNA sequence that codes for the AR protein (Chang *et al.* 1988; Lubahn *et al.* 1988; Trapman *et al.* 1988; Tilley *et al.* 1989). The human androgen receptor is a monomeric protein of between 910 and 919 amino acids (with variation caused by polymorphism of the protein at the N-terminal region, see later). The molecular weight of the receptor is approximately 100-110 kDa. Three important structural domains with different functions are recognised, in a similar way to other human hormone receptors. There are three basic functions of AR recognised; ligand binding, DNA binding and transcriptional activation (transactivation). In addition, other functions such as nuclear localization signals (NLS) regions and a hinge area (believed to be important in nuclear localization and for interactions between the steroid-binding and transcriptional activating domains of the receptor) are also recognised.

Ligand binding within the AR occurs in the COOH-terminus domain of the protein and this is believed to be only one of several ways of conferring specificity for action as the AR. The ligand-binding domain of the molecule is approximately 30 kDa and 253 amino acids in length. It is predominantly hydrophobic and contains a high

methionine content that may be important for ligand binding (Prins 2000). Recent crystallography studies of the AR LBD have shown considerable similarity in the molecule's 3-dimensional structure, compared with many of the other steroid receptor superfamily (Gelmann 2002). The AR LBD is folded into 12 helices that form a ligand-binding pocket. Helices 4, 5 and 10 are the primary contact regions of the AR with its ligands. At androgen binding, helix 12 folds over, enclosing the ligand, and discloses a groove that binds to the N-terminal domain of the molecule (Gelmann 2002).

It appears unlikely that only a single function is subserved by the ligand-binding domain and ligand binding should not be thought of as a simple one step process. Prior to androgen binding, this area of the AR appears to be able to act as an inhibitor domain, preventing the receptor from activation in the absence of ligand (Hiort *et al.* 1998). The ligand-binding region has also been implicated in interactions with HSPs, receptor dimerisation and ligand-binding dependent phosphorylation, and also contains an important ligand-dependent receptor transactivation sequence (known as AF-2, 884-Glu-Met-Met-Ala-Glu-888) (Brinkmann *et al.* 1999). This transactivation function is hormone-dependent and weaker than in other steroid receptors, but can be strongly enhanced by nuclear receptor co-activators (Brinkmann *et al.* 1999; Taplin *et al.* 2001).

Evidence for which amino acids are important for ligand binding has come from deletion and mutation studies, androgen-resistance syndromes and from prostatic tumours. Broadly, deletions of > 10 amino acids are known to severely affect

androgen binding. In contrast if the entire ligand-binding domain is deleted, the remaining molecule is constitutively active (Brinkmann *et al.* 1999).

The central area of the AR protein is known to be the region of DNA binding. This domain has the highest degree of structural conservation within the human steroid receptors family (Lubahn *et al.* 1988), with approximately 80 % homology with human GR and PR.

The DNA binding domain is the smallest of the three main structural domains at approximately 10 kDa, 68-amino acids in length and is rich in cysteine residues. Four of the total nine cysteine residues bind one zinc ion each, allowing the domain to fold into two type II zinc-finger patterns, conferring the ability to bind the major groove of DNA (Gelmann 2002). The two zinc fingers incorporate two perpendicularly orientated α -helices and specific amino acids (glycine, serine and valine) in the α -helix contact the DNA. These are believed to define the specificity of the receptor for its hormone response element across a number of the human steroid receptor family (Brown 1995).

The first zinc finger mediates specific receptor-DNA interactions (Hiort *et al.* 1998) by a group of distal amino acids known as the P box, important for sequence recognition. The second zinc finger appears to be involved in the receptor dimerisation necessary for DNA binding (Hiort *et al.* 1998), and contains a 'D box' group of amino acids at its proximal end, important for homodimerisation of the AR (Brown 1995; Prins 2000). The second zinc finger stabilizes the AR-DNA complex and has also been implicated as part of the nuclear localization signal site and

receptor dimerization, together with an area that passes into the hinge region (Jenster *et al.* 1991; Simental *et al.* 1991; Jenster *et al.* 1993; Prins 2000; Gelmann 2002).

The NH₂-terminus of the AR protein is the largest at approximately 556 residues and is the domain implicated in transactivation. The greatest variability in amino acid sequence within the receptor occurs in this region, as in other classes of human steroid receptors. Polyglutamine, polyglycine and polyproline repeat groups of approximately 20 residues each are found in this domain (Taplin *et al.* 2001). These areas are polymorphic in length within the normal human population. The polyglycine trimeric (CGN)_n repeat appears to be unique to the AR (Brown 1995) and the exact function of these repeat sequences is unknown. Increasing length of the polyglutamine tail has been implicated in diseases such a X-linked spinal and bulbar muscular atrophy (Kennedy syndrome) and prostate cancer (Brown 1995; Nam *et al.* 2000). The age-related decline in serum androgen levels in men has also been linked to increasing CAG repeat sequences in exon 1 (Krithivas *et al.* 1999). A shorter polymorphic CAG repeat is associated with a higher transactivation of the AR (Krithivas *et al.* 1999; Prins 2000) and benign prostatic hyperplasia (Nelson *et al.* 2002). The full function of these repeats has not been elucidated but in other proteins with similar sequences, such features are implicated in the control of gene expression (Gerber *et al.* 1994). Inhibition of the nuclear receptor co-activator p160 has been shown to occur with increasing AR polyglutamine length further suggesting a role in control of gene expression (Irvine *et al.* 2000).

The N-terminus domain of the receptor also contains a high concentration of acidic amino acids between residues 100-325, resulting in a net negative charge in this area.

The significance of this finding is not yet clearly understood. The exact boundaries of the NH₂-terminal transactivation domain (designated as AF-1) are not yet defined for the human AR. A region between amino acids 51-211 is known to be essential for transactivation in the full length human AR (Brinkmann *et al.* 1999). Evidence for two different transactivation domains termed AF1 and AF5 in the NH₂-terminal domain that depend on the form of the receptor protein have been reported (Berrevoets *et al.* 1998; Langley *et al.* 1998). These groups have also provided evidence for possible interactions between the ligand-binding domain and the transactivation domains of the NH₂-terminal domain in human AR protein (Berrevoets *et al.* 1998; Langley *et al.* 1998). More recently, the N-terminus domain has been recognised as the primary site for interaction of AR with co-activators that amplify the transcription signal (Gelmann 2002).

Phosphorylation of the AR appears to principally occur in the NH₂ transactivation domain but the exact role of all phosphorylation steps has not been fully elucidated. The AR is a phosphoprotein, and phosphorylation of serine residues has been found to be required for acquisition of ligand-binding activity (Blok *et al.* 1998). Substitution of the serine residues within the NH₂-terminus domain has, however, been shown to have no significant effect on transcriptional activity (Jenster *et al.* 1994).

Mechanisms of Androgen-Dependent Gene Transcription.

The basic mechanism proposed for hormone-regulated gene transcription involves the activated nuclear receptor directly or indirectly recruiting and maintaining a pre-initiation transcription complex at the promoter site of the target gene (McKenna *et al.* 1999). The exact mechanisms by which nuclear receptors and cofactors interact resulting in tissue-specific gene regulation remain unclear. The basal transcription apparatus (general transcription factors, GTF) are believed to be partly pre-formed as a stable pre-initiation complexes within the nucleus. Components of the GTFs include the enzyme RNA polymerase II (RNA Pol II), the multiprotein transcription factor II δ (TFIId), composed of TATA binding protein (TBP) and a number of TBP-associated factors (TAF_{II}s), and the transcription factors II β (TFIId), and II δ - α (TFIIf- α). Transactivators (such as the AR homodimer) are thought to stimulate gene expression by facilitating the assembly of the basal transcription factors into these stable pre-initiation complexes at the hormone-regulated promoter site, thereby increasing the rate of RNA synthesis (Shibata *et al.* 1997; McKenna *et al.* 1999). Steroid receptors are known to be able to directly interact with components of the basal transcription complex such as the TBP and transcription factor II β (TFIId) (Shibata *et al.* 1997). Recruitment of TFIId into the basal transcription-DNA complex is a rate-limiting step in the formation of the transcription pre-initiation complex, and interaction of nuclear receptors with TFIId may stabilize or assist this process.

Interactions with Sequence-specific Transcription Factors

The control of gene promoters by steroid hormone receptors is also known to require sequence-specific transcription factors as well as the general transcription factors mentioned above. These sequence specific factors are important for the efficient activation of promoter regions and can result in activation or repression of promoter activity. The sequence-specific transcription factor AP-1 (a heterodimer that can be made up of various combinations of c-Jun and c-Fos,) is known to regulate transcription by binding directly to AP-1-responsive genes. The AP-1 transcription factor acts to translate signals induced at cell membrane receptors, and is transduced by the protein kinase signalling cascades. The interaction of GR with AP-1 has been studied extensively and has been considered to be the paradigm of transcriptional cross talk (Gottlicher *et al.* 1998). The interaction of AP-1 with target gene promoters is believed to involve only a single binding site for either AP-1 or GR in the promoter region. This interaction of AP-1 and GR results in the repression of AP-1-induced gene transcription. Repression appears to be a mutual process, as AP-1 overexpression represses the activity of steroid receptors on promoters containing GH-response elements. A similar interaction has been shown for interactions of AP-1 with the AR (Shemshedini *et al.* 1991).

The c-Jun component of AP-1 has also been shown to be able to interact with the DNA-binding domain and hinge region of the AR, and mediate AR-dependent transactivation (without DNA binding or interaction with c-Fos, Bubulya *et al.* 1996).

Co-activators of Steroid Receptors (see **Figures 1.1 and 1.2**)

Nuclear receptor co-activators are believed to regulate transcription by controlling interactions between the basal transcription machinery, sequence-specific transcription factors, and the chromatin environment. One of the first pieces of evidence for this process came from the demonstration of a phenomenon known as ‘squelching’ (Meyer *et al.* 1989). In this process, overexpression of one receptor results in the inhibition of either its own or other steroid receptor activity. This suggested the possibility that other factors may also modulate transactivation due to a limiting amount of an undefined nuclear co-activator(s). The first prototypical steroid co-activator identified was steroid receptor co-activator 1 (SRC-1, Oñate *et al.* 1995), although other proteins such as a 160 kDa ER-associated protein (ERAP-160), a 170 kDa GR-interacting protein and several other receptor interacting proteins (RIP-80, RIP-140 and RIP160) had been identified before this, when their full function was not known (McKenna *et al.* 1999). SRC-1 is known to interact with and co-activate nuclear receptors, interact with other nuclear co-activators, contact basal transcription factors and possess chromatin modifying (histone acetyltransferase) activity. An SRC family of co-activators has been identified based on sequence and functional similarity to SRC-1. Redundancy of action within this family, as indicated by SRC-1 knockout mice studies, has already been identified (McKenna *et al.* 1999).

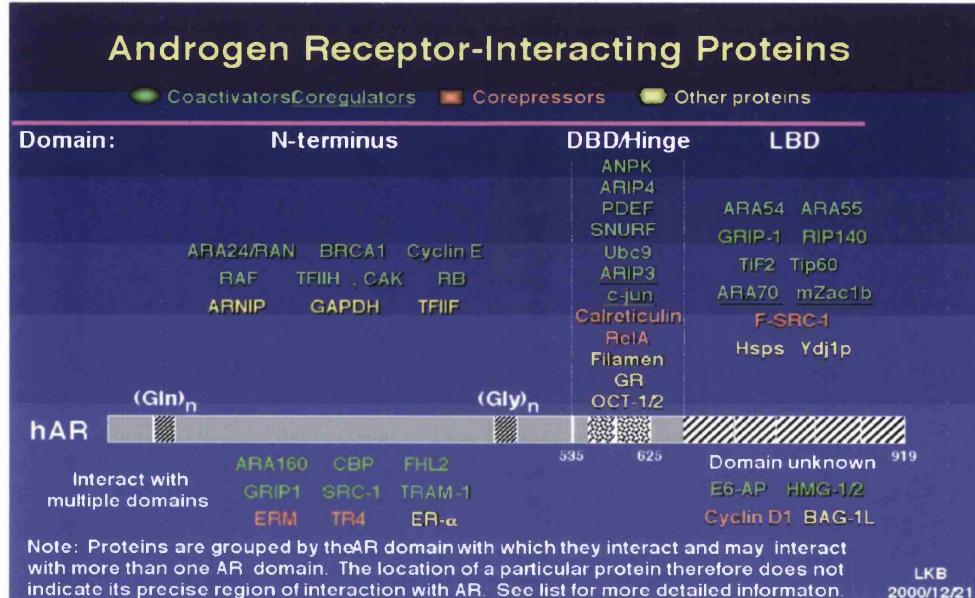
Since the initial characterisation of SRC-1, considerable evidence for a large number of other nuclear receptor co-activators has accumulated, with over twenty being listed by McKenna *et al.* (McKenna *et al.* 1999a). Further interactions of co-activators with other molecules as well as the AR continue to be identified. The

important principle for this group of molecules is that multiple types and modes of action of co-activators appears to be in keeping with the complex and tightly regulated nature of the gene transcription. The nomenclature of nuclear receptor co-activators is complex and a unifying nomenclature has been proposed by Li and Chen (Li and Chen 1998).

Co-repressors of Steroid Receptors

Generally less is understood about the co-repressors of steroid hormone transactivation compared with the co-activators described above, and the specific role of co-repressors in androgenic pathways remains poorly described. Discrete co-repressor complexes in mammalian cells have however been identified (Torchia *et al.* 1998). A number of co-repressor proteins have initially been characterised such as nuclear receptor co-repressor (NCoR), the related silencing mediator for retinoid and thyroid hormone receptor (SMRT), thyroid receptor uncoupling protein (TRUP) and small ubiquitous nuclear co-repressor (SUN-CoR) (McKenna *et al.* 1999). There is evidence that some of these molecules, such as NCoR and SMRT, may be included in the general mechanisms of active transcriptional repression used by other classes of transcription factors (Torchia *et al.* 1998).

Figure 1.2



From the androgen receptor gene mutation database, www.mcgill.ca/androgendb/

Drawn by Dr L.K. Beitel

Chromatin Structure and Regulation of Hormone Receptors

Efficient transcription requires the cellular ability to reorganize chromosomal material within the nucleus to a state where transcription can progress. DNA within chromosomes is organised into DNA–protein nucleosome units, with the basic nucleosome made up of histones, small, highly basic globular proteins that directly associate with DNA. This organisational hierarchy results in a barrier to transcription factors accessing DNA. Regulation of nucleosome and histone structure is another important way that transcriptional control is achieved.

In nuclear receptor transactivation, the binding of activated receptors is believed to result in de-repression, the modification of local chromatin structure that allows transcription to occur. De-repression is then followed by activation and recruitment of general transcription factors to form the preinitiation complex at the promoter site. Nuclear receptor co-regulators (activators and repressors) are thought to have both the intrinsic ability to modify chromatin and the ability to recruit chromatin-modifying proteins to enable transcription to occur (McKenna *et al.* 1999). Access of transcription factors to DNA is thought to be regulated by two classes of molecules, the histone acetylases and deacetylases. Generally, histone acetylation is associated with increased transcriptional activity and deacetylation with transcriptional repression. A number of nuclear receptor co-activators have been shown to have histone acetylase activity as well as some elements of the general transcription machinery such as TFIID (McKenna *et al.* 1999). Acetyltransferases have also been implicated in the acetylation of non-histone proteins, and this has been interpreted as

implying a broader role for acetyltransferases in cell signalling (McKenna *et al.* 1999). However to date, information on the direct role of acetylation in nuclear hormone receptor function is still scarce.

To summarise, control of nuclear steroid receptor transcriptional activity is a complex, multi-step process, which is only now beginning to be partly understood. The layers of regulation emerging requires the action of enzymes and co-regulators, organised into prearranged complexes, allowing multiple configurations to occur. Co-regulators interact with other classes of molecules (GTFs, sequence specific transcription factors and chromatin) at the site of gene promoters. This also allows for multiple sites of transcriptional control, necessary to prevent unregulated transcription, and allow a measured reaction to ligand signalling within target cells.

1.3.3 Human Androgen-Responsive Genes

More than 100 androgen-regulated genes are known today, and many more are likely to be discovered (Koivisto *et al.* 1998). Important cell cycle control and signalling molecules such as cyclins A, D1-3, E, cyclin dependent kinases, Fos and Jun, the intracellular signal transduction genes for Ha-ras and p21, peptide growth factors and their receptors have been shown to have some degree of androgen-responsiveness (Koivisto *et al.* 1998).

The actions of androgens in a number of different tissues may be either stimulatory (the majority of cases known) or inhibitory. Molecular cloning and sequencing of some known androgen-responsive genes has led to the characterization of androgen-

responsive elements (ARE) within them. Androgen-responsive elements are DNA sequences that are responsible for androgen-dependent gene transcription (see **Table 1.1**, adapted from Chang 1995).

Most AREs identified have been in animal (usually mammalian) models. Corresponding data on human genes containing AREs is more limited. For example, a number of prostatic proteins such as PSA, GKLK2 protease, prostate specific membrane antigen and prostatic acid phosphatase are well known to be under androgenic control in humans and other mammals. The TR2 and TR3 orphan receptors, initially identified from human prostate and testis, can suppress or induce respectively by androgens at an mRNA level. Other organs of the male genital tract such as the seminal vesicles, vas deferens and epididymis have also been shown to express androgen-dependent proteins in rat and mouse models.

Table 1.1 Examples of known ARE sequences for selected genes.

Gene	Sequence
PSA (Glandular Kallikrein-3)	5'-AGTCACTGCTGTTCT-3'
Ornithine decarboxylase	5'-AGTCCCAC TTGTTCT-3'
β -Glucuronidase	5'-AGTACTTGTGTTCT-3'
Mouse Vas Deferens Protein	5'-TGAAGTT CCTGTTCT-3'
Factor IX	5'-AGCTCAGCTTGTACT-3'
Glandular Kallikrein-2	5'-GGAACAGCAAGTGCT-3'
Probasin	5'-ATACGATCTTGTCT-3' and 5'-GTAAAGTACTCCAAGAA-3'

By performing a 'Blast' search for such sequences in on-line nucleotide sequence libraries, it is possible to identify identical or near-identical base sequences with the aim of distinguishing other potential androgen-responsive genes. For example, using the U.S. National Centre for Biotechnical Information (NCBI) database (National Library of Medicine, National Institute of Health, <http://www.ncbi.nlm.nih.gov/BLAST/>) and inputting a known ARE sequence, the number of identified genes containing this type of ARE can be found. A search with the ARE sequence 5'-AGTACGTGATGTTCT-3' listed in the table above (Factor IX) shows no other identical nucleotide sequences have been found for any other human genes (implying this ARE is currently unique to this gene). Other sequences with near homology (e.g. 14/15 identical bases) that are also retrieved can also be

reviewed to see if their associated proteins are known or suspected to be androgen-related.

By inputting the search term 'androgen response element' into the same database, a total of seventeen androgen-responsive sequences were retrieved (as of 23.04.03). Eight of these are of human origin; six include AREs within specific genes (plus one for calreticulin mRNA, a known androgen-responsive multi-functional calcium-binding protein, and one for the cAMP-response element of the human androgen receptor gene promoter).

1.4 The Androgen Receptor In Androgen-Insensitivity Syndromes.

Mutations in the androgen receptor gene may result in the production of abnormal androgen receptor protein. This in turn may lead to Androgen-Insensitivity Syndrome (AIS), where a 46 XY male has androgen-related male sexual growth and maturation impaired to a variable degree. If there is complete androgen insensitivity, (CAIS, also known as complete testicular feminisation) both the internal and external reproductive tract develops into a complete female phenotype. Partial AIS (PAIS) can produce a spectrum of genital tract development that ranges between normal males (normal fertility with or without under-virilization of secondary sexual characteristics), infertile males, men with Reifenstein syndrome (gynaecomastia and hypospadias), and near-normal female (incomplete testicular feminisation). Nomenclature for these disorders has been variable, and a classification system based

on external phenotype has been produced (known as AIS types I-V) using a similar system to the congenital adrenal hyperplasia classification (Hiort *et al.* 1998).

To date, over 500 AR mutations have been reported in AIS. These mutations are collated as the AR Gene Mutation Database by workers at McGill University (Gottlieb *et al.* 1999), with information accessible via the World Wide Web (<http://www.mcgill.ca/androgendb/>). At their last published report, 374 of the mutations were of germline origin, with a smaller number of somatic mutations known. These mutations have only been found in 5 disease states (AIS in all its forms, prostate cancer, breast cancer, laryngeal cancer and Kennedy syndrome), as well as a small number of mutations reported from normal individuals.

Defects in the AR are much more frequent than in other members of the type I steroid receptor class. Three features regarding the AR have been postulated to account for this observation (McPhaul *et al.* 1993). The anatomical and functional abnormalities that result from AIS during embryogenesis and puberty lead to a high rate of discovery of AR abnormalities. Androgenic action is not required for foetal viability and individuals with severe AR dysfunction without the capacity to reproduce can still be born. Finally as the gene for AR is X-linked, only one copy of the AR gene is available and the XY genotype constitutively expresses all mutations.

There appears to be no precise relationship between the clinical phenotype of a patient with AIS and the character of the AR abnormality as defined by both qualitative and quantitative assays of androgen binding. Such studies have shown

that indistinguishable functional bioassays may be associated with an undervirilization phenotype in some families yet cause CAIS in other families.

Certain areas of the AR appear to be associated with mutation 'hotspots' in both AIS and prostate cancer. In AIS, a hot spot of 48 mutations in exon 5 between amino acid positions 724 and 772 exists (Gottlieb *et al.* 1999).

Future developments in the understanding of AIS will come with increasing knowledge of the nature of the normal AR, further characterization of the mutations that affect it and its interactions. Developments in this field may also turn out to have applications in increasing our understanding of prostatic disease such as prostatic carcinoma.

1.5 The Androgen Receptor in Prostate Cancer

1.5.1 Introduction

Prostate cancer is a common malignancy amongst men and is one of the leading causes of death from malignancy in the Western World (Metlin 1997). In 1993, in England and Wales 17,210 new cases were diagnosed and during the period 1971-1993, the incidence of the disease increased by 179% (Majeed *et al.* 2000). The disease remains a considerable burden on the National Health Service.

Early stage prostate cancer may be cured by prostatectomy or radiotherapy but a proportion of these patients will relapse, ultimately with incurable disease. In addition, a significant proportion of patients in the United Kingdom still present with

metastatic disease. For men with advanced or metastatic disease, cure is unlikely, and androgen deprivation is the mainstay of treatment. Many of these approaches are known to act directly or indirectly through the androgen pathway (e.g. surgical or medical castration, steroid and non-steroidal anti-androgen therapy). However, despite the high initial response rate to androgen deprivation (up to 80%), relapse and progression to hormone-refractory prostate cancer (HRPC) occurs with a median time of eighteen months (McLeod *et al.* 1997). Once HRPC occurs, median survival for this stage of disease is approximately six months (Porter *et al.* 1993; Logothetis *et al.* 2000).

There is a close inter-relationship between the development of bone metastases and subsequent HRPC, with complications from secondary skeletal spread dominating the symptoms from metastatic prostate cancer (McCrea *et al.* 1958) and skeletal metastases being the commonest site of hormone-refractory disease (Galasko 1981). A direct relationship between the extent of osseous involvement and survival in HRPC has been demonstrated (Soloway *et al.* 1988; Sabbatini *et al.* 1999). Only limited palliative treatments are possible for metastatic bone disease (e.g. second-line hormone therapy, radiotherapy, chemotherapy or surgery) and currently, there is no real effective treatment for HRPC. Better therapies are clearly required. The molecular mechanisms that underlie bone metastases in prostate cancer and the development of hormone-refractory disease in bone and other sites need to be understood.

1.5.2 Manipulation of the AR pathway as Treatment for Prostate Cancer

Androgens are generally believed to play a crucial role in both the pathogenesis and progression of prostate cancer, which is one of the most hormone dependent of all tumour types. However, there still remain many unanswered questions about the exact role of androgens in the development and progression of the disease. Evidence for a role of androgens in the pathogenesis of prostate cancer comes from several sources. A causal relationship between androgens and prostate cancer seems plausible because the malignancy develops in androgen-dependent epithelium and is usually androgen sensitive. However there is not convincing epidemiological evidence for an association between circulating hormone concentration and prostate cancer risk at either a population or case-control level (Bosland 2000, Gelmann, 2002). The best current hypothesis suggests that androgens act as strong tumour promoters, via androgen receptor-mediated mechanisms on a background of endogenous genotoxic carcinogens and possibly weak environmental carcinogens. (Bosland 2000). Previously suggested risk factors for the development of prostate cancer have included increased availability of androgens and altered cellular availability of androgens (Labrie *et al.* 1993; Kallioniemi *et al.* 1996). Several studies have suggested that eunuchs, castrated before puberty, or men castrated before the age of 40 do not develop prostate cancer or benign prostatic hypertrophy in later life (Zuckerman 1936; Moore 1944; Jie-ping *et al.* 1987).

Since the pioneering work of Huggins and Hodges in the 1940s, patients with locally advanced or metastatic disease are usually treated initially with androgen deprivation therapy in order to suppress the growth of the tumour and improve symptoms from

local or metastatic disease (Huggins *et al.* 1941). Increasingly, many patients with early stage prostate cancer treated with curative modalities such as radiotherapy are also being offered androgen deprivation therapy as a standard part of their treatment (MRC Radiotherapy Working Group 2000).

Endocrine therapy for prostate cancer can be achieved by a number of methods, all of which interfere with the androgen receptor pathway. Historically, cessation of androgen production in the testes has been achieved by castration, leading to a rapid fall of serum androgen concentration after a short time. This treatment probably remains the gold standard for androgen deprivation. However, in the early 1980s, LHRH agonists such as leuprolide and goserelin were introduced in clinical practice. These agents produce a similar level of fall in serum androgen levels to orchidectomy, but after a longer period of time (weeks rather than days). Initially, a rise in serum testosterone levels is seen as the agonist analogue produces a supraphysiological release of LH and FSH. This is followed by down-regulation of the pituitary gonadotrophin cells receptors causing the decline in serum testosterone to castrate levels. Both of these methods act on the AR pathway by directly diminishing levels of ligand for the AR. Recently, LHRH antagonists have also been synthesised and undergone phase III evaluation (Taplin *et al.* 2001).

The function of androgens in peripheral tissues can also be blocked by anti-androgens. These drugs, usually competitive inhibitors of the androgen receptor, interact with the AR ligand-binding domain and are also widely used in the treatment of prostate cancer. The precise nature of antiandrogen-LBD interactions is also important in the study of mutations within the androgen receptor as discussed in the

next section. Anti-androgens are sub-classified into steroidal anti-androgens, such as cyproterone acetate and the nonsteroidal anti-androgens, such as flutamide, nilutamide and bicalutamide. Following castration, antiandrogens are able to competitively block the effects of the weaker serum adrenal androgens, androstenedione and dihydroepiandrosterone. Prior to castration, if used as monotherapy, nonsteroidal anti-androgens produce a 30-60% rise in serum testosterone and may not completely block all androgen action.

The combination of surgical or medical castration and anti-androgen therapy, known as maximal androgen blockade (MAB) has been previously suggested as a logical and effective endocrine manipulation of the AR pathway for the treatment of prostate cancer. However, such treatment is widely now believed not to have fulfilled its' initial promise (de Voogt *et al.* 1998). A recent large meta-analysis has suggested only limited possible benefits over single therapy treatment in thousands of men treated with MAB (Prostate Cancer Trialists' Collaborative Group 2000).

Molecular and histological responses of prostate cancer to androgen deprivation have mainly been studied in model tumour and cell line systems. The most typical feature of androgen deprivation is a decrease in cellular growth rates. Apoptosis (as measured by the tumour apoptotic index) has also been shown to occur in response to androgen ablation. In the human prostate cancer PC-82 athymic nude mouse model, both of these two changes were seen (Kyprianou *et al.* 1990).

With increasing time, other previously noted histological changes in human prostate tumours in response to androgen deprivation have included reduction in tumour,

glandular, nuclear and nucleolar size, cytoplasmic vacuolation and nuclear pyknosis (Mostofi *et al.* 1992). There may be significantly intra-tumoural and inter-tumoural variability in the degree of these responses. In approximately one-third of well-differentiated and two-thirds of poorly differentiated tumours, little apparent change in tumour morphology is seen after androgen withdrawal (Murphy *et al.* 1991). In a study of the short-term effects of therapeutic castration on human prostate carcinomas *in vivo*, Westlin *et al.* studied 18 biopsies taken 1 day before and 7 days after castration. In only 3 tumours were there no changes in histological appearance. Histological changes detected were vacuolation of the tumour cell cytoplasm, decrease in nuclear size and reduced Ki-67 proliferative index. Apoptotic index remained unaffected in 12 of the cases, suggesting that rapid induction of apoptosis occurs in only a minority of human prostate cancers (Westlin *et al.* 1995).

Amongst the earliest effects of androgen withdrawal seen in tumour model systems are decreased intranuclear concentrations of AR and DHT. This is believed to produce conditions where androgen stimulated genes become quiescent and androgen-repressed genes can become activated (van Doorn *et al.* 1976).

1.5.3 Mechanisms for Prostate Cancer cells to become Independent of the Androgen Pathway

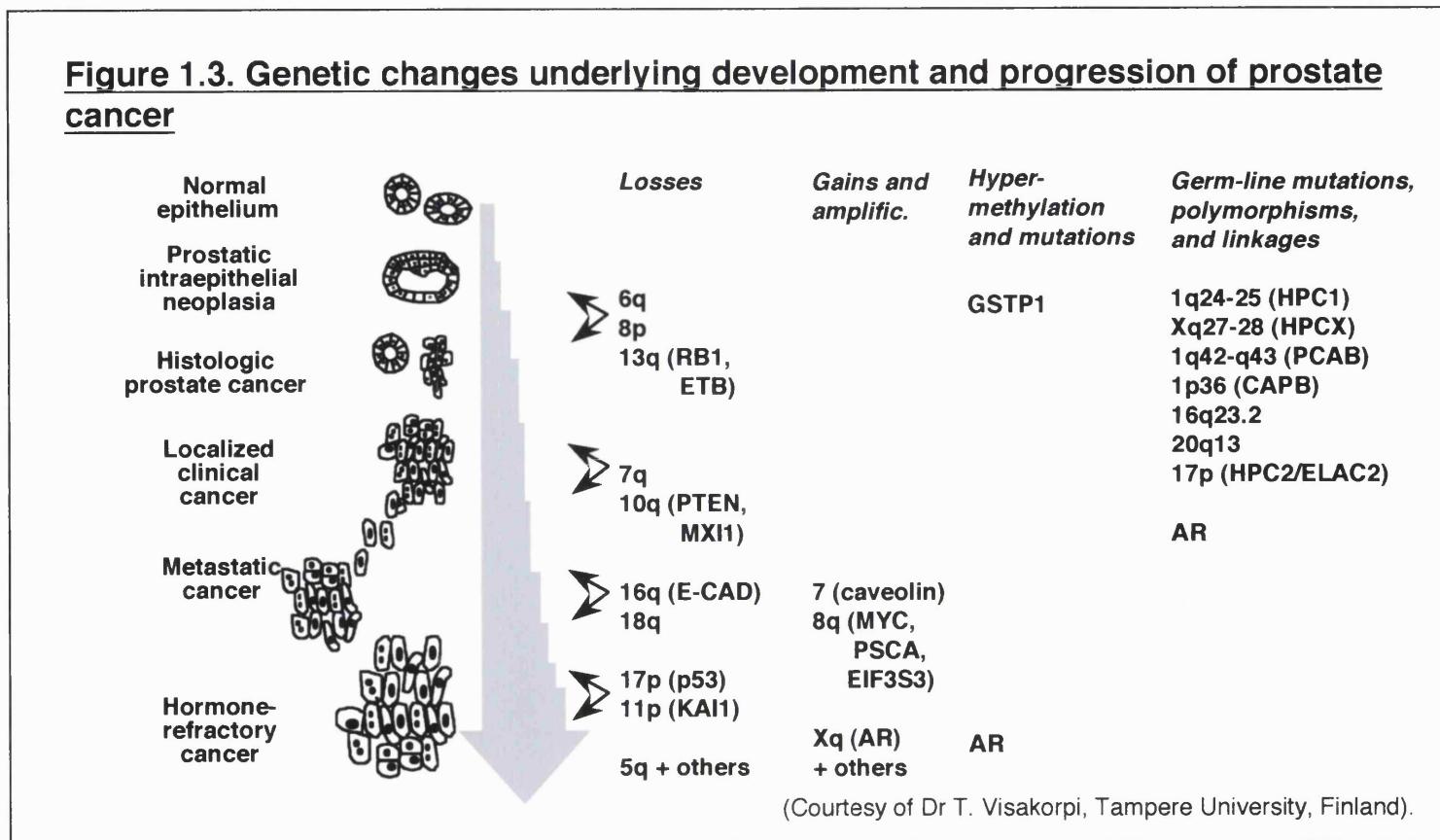
Bypassing the AR Pathway, AR-ligand independent activation and synergistic activation of the AR.

Prostate cancer cells strongly respond to androgens but growth is not regulated by these molecules alone, with tumour growth the result of many stimulatory events. It is likely that both the activation of oncogenes and the inactivation of tumour suppressor genes contribute to hormone-refractory prostate cancer. A number of steps in the development and molecular progression of prostate cancer have already been partly characterised (see **Figure 1.3**). Changes to the androgen pathway are only one part of this (Visakorpi *et al.* 1995; Elo *et al.* 2001). The transition to an androgen-independent phenotype is a multi-step process (Vogelstein *et al.* 1993).

It was long believed that only steroid hormones activated their respective receptors to initiate transcription of steroid-responsive genes. A number of recent studies now provide evidence for coupling of other signal pathways of various cellular regulators to steroid hormone receptors. Androgen-independent activation of the AR and synergistic activation of AR in the presence of low levels of androgens have both been recognised. These findings provide an attractive hypothesis for mechanisms involved in the development of the androgen-independent phenotype. Several different classes of molecules or pathways have been shown to be involved in AR-ligand independent or synergistic activation. These include the peptide growth

factors, insulin-like growth factor-1, epidermal and keratinocyte growth factors (Culig *et al.* 1994), interleukin-6 (Hobisch *et al.* 1998), cell signalling molecules such as cAMP (Ikonen *et al.* 1994; Culig *et al.* 1997) butyrate and bombesin (Taplin *et al.* 2001), protein kinase A (Nazareth *et al.* 1996), protein kinase C (de Ruiter *et al.* 1995), mitogen-activated protein kinase (Zhu *et al.* 1997; Yeh *et al.* 1999; Eder *et al.* 2001), c-ErbB2 / HER-2 (Craft *et al.* 1999) and hormones such as LHRH (Culig *et al.* 1997). Such interactions have been termed 'cross talk', and evidence for this is increasing rapidly. Cross talk appears to be involved in autocrine growth stimulation. Autocrine growth signalling is important in tumour cell growth and progression, and many prostate cancer cells express growth factors and their receptors that are not normally produced, or produced only in low levels, by normal prostate epithelium (Russell *et al.* 1998). Synergistic activation of the AR, by allowing a lower concentration of androgen needed for maximal AR activation may be especially important in patients with advanced prostate cancer in which serum androgens are suppressed by therapy.

Figure 1.3. Genetic changes underlying development and progression of prostate cancer



One of the most well characterised examples of cross talk between the AR pathway and growth factors is the interaction of AR and insulin-like growth factor-1 in tumour cell lines. Insulin-like growth factor-1 is a potent mitogen for many tumours. The androgen-sensitive LNCaP cell line, which contains endogenous AR, was used to demonstrate an effect of growth factors on the expression of the androgen-regulated PSA gene, as measured by PSA secretion into the tissue culture supernatant (Culig *et al.* 1994). Insulin-like growth factor-1 increased supernatant PSA levels 5-fold, and was only slightly less effective than a synthetic androgen methyltrienolone (Culig *et al.* 1994). To confirm that insulin-like growth factor-1 was not acting independently on the AR, PSA production was blocked by the addition of the non-steroidal antiandrogen, bicalutamide, to the conditioning medium. In another cell line, DU-145 (which does not natively express AR), transient transfection with an androgen-inducible reporter gene and an AR expression plasmid, insulin-like growth factor-1, epidermal and keratinocyte growth factors all activated the AR, but to different degrees (Culig *et al.* 1994).

Forced overexpression of c-ErbB2 / HER-2 in the LAPC-4 prostate tumour cell line resulted in ligand-independent activation of the androgen receptor pathway in a study by Craft *et al.* 1999. In CV-1 tumour cells, cAMP, forskolin and phorbol 12-myristate 13 acetate (both activators of protein kinase A) all caused synergistic effects with testosterone. Synergism was antagonized by bicalutamide. Cyclic AMP in CV-1 cells also appeared to be a weak stimulant of reporter-gene activity without testosterone. Taken together, these findings show that the AR may be activated in a ligand-independent and synergistic manner (Craft *et al.* 1999).

The complete bypassing of the androgen receptor has also been achieved in tumour cell lines *in vitro*. In LNCaP tumour cells, androgen-resistance can be created by transfection of the ras or bcl-2 oncogenes (Voeller *et al.* 1991; Raffo *et al.* 1995).

These initial studies show that the full pathways of androgen-independent AR activation and synergistic action via cross talk have not yet been fully characterised but are becoming increasingly well understood. This may provide further insights into the development of the androgen-refractory phenotype of advanced prostate cancer.

Adapting the AR pathway to low levels of Androgens

Mutations in the AR Gene and Receptor in Prostate Cancer

Mutations in the AR were first described in androgen insensitivity syndromes and such inactivating mutations are rare in prostate cancer (Nazareth *et al.* 1999). The first *in vivo* AR gene mutation in prostate cancer was found in 1992 (Newmark *et al.* 1992), two years after mutation in the LNCaP cell line were detected (Veldscholte *et al.* 1990). Since then, over 75 further different mutations have been reported in prostate cancer (compared with over 530 in androgen insensitivity, <http://www.mcgill.ca/androgendb/>).

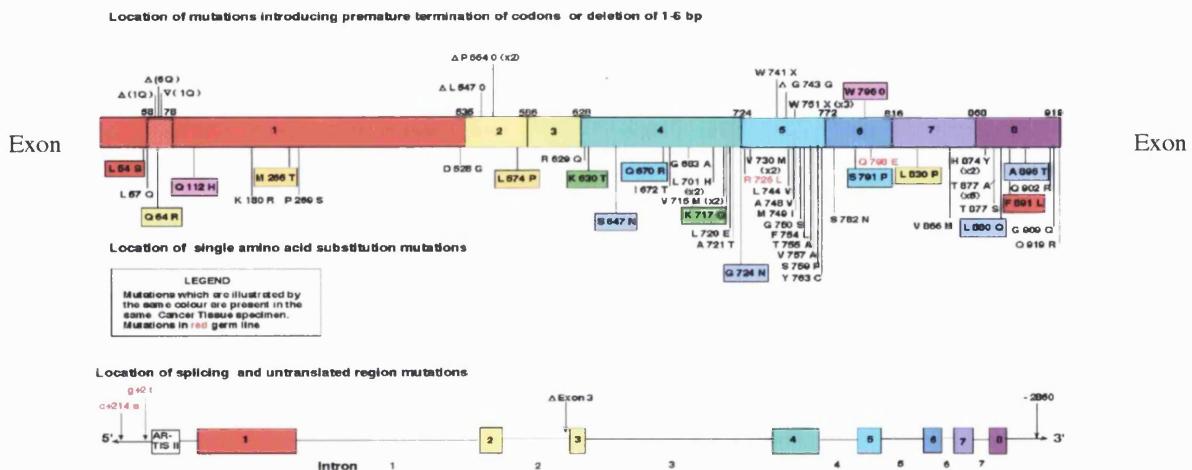
Most mutations in prostate cancer are single amino acid substitutions and the functional consequences of these have not been studied in detail. Other types of mutations such as deletion and insertions have also been observed. A small number

of germ line mutations in prostatic tumours also occur. Many mutations do not appear to affect AR function *in vitro*. Within the AR gene, mutations in prostate cancer have been most commonly found in the ligand-binding domain (see Figure 1.4), although all eight exons have been shown to be affected. A cluster of mutations occurs within the LBD in a loop between helices 3 and 4, in common to a number of other steroid receptors (Gelmann 2002). A second cluster of mutations also occurs in the LBD, at amino acid positions 874-910. This may affect binding of both steroid co-activator molecules and the N-terminus domain (Gelmann 2002).

In a number of cases, ligand-binding domain mutations lead to altered receptor-protein interactions and receptor transactivation by steroids other than androgens (e.g. progesterones, oestrogens or anti-androgens). These mutations in the AR gene appear to underlie the 'androgen-withdrawal response', where cessation of anti-androgen therapy paradoxically results in a further clinical improvement in symptoms and a drop in serum PSA. To date, reports of this phenomenon have been reported with all anti-androgens (Scher *et al.* 1993; Nieh 1995; Suzuki *et al.* 1996). Mutations of this type are generally rare in primary tumours and more common in late-stage disease (i.e. metastatic and HRPC tumours). A high frequency of such AR mutations (eight mutations in five tumours from ten patients) was reported in bone metastases from HRPC in a study in 1995 (Taplin *et al.* 1995). This same group have also recently suggested that selection for such AR mutations in advanced prostate cancer occurs as a consequence of combined anti-androgen treatment with flutamide and castration (Taplin *et al.* 1999). In five of sixteen patients who received flutamide, mutant ARs were found, all strongly stimulated by flutamide. In contrast, only a single mutation in the AR gene was found amongst seventeen patients who received

monotherapy with androgen-ablation alone. Patients whose mutant AR was stimulated by flutamide responded to further anti-androgen therapy with bicalutamide, suggesting a molecule-specific mechanism of action.

Figure 1.4 ANDROGEN RECEPTOR GENE MUTATIONS IN PROSTATE CANCER.



From the androgen receptor gene mutation database, www.mcgill.ca/androgendb/

Recently, the role of CAG and CCG trinucleotide repeat sequences in the AR gene has been of interest in prostate cancer. Short glutamine repeat-sequences appear to predict for a higher risk, higher grade and more advanced stage of prostate cancer at diagnosis and for earlier onset of disease (Stanford *et al.* 1997; Kantoff *et al.* 1998; Gelmann 2002; Nelson *et al.* 2002). A shorter glutamine-repeat sequence also results in an increase in AR transactivation, suggesting the higher the activity, the higher the risk for prostate cancer (Jenster *et al.* 1994). However, not all studies have suggested these findings are universal (Nam *et al.* 2000; Beilin *et al.* 2001). What is particularly interesting about AR gene polymorphisms is that, in contrast to rare highly penetrant mutations in genes such as p53 or BRCA-1 (that confer a very high risk on a few individuals), the AR CAG repeat polymorphism may confer variable risks upon many individuals.

Androgen Receptor Co-activators and Co-repressors in Prostate Cancer

The role of co-regulators of the androgen receptor in prostate cancer is now attracting increasing attention. It is now accepted that activation of steroid receptors such as the AR is dependent upon interactions with receptor-specific or general co-regulators (Taplin *et al.* 2001). Investigations remain in the early phase, with relatively little known about the complex pathways that control steroid-activated gene transcription. Enhancement of receptor activity by the amplification of steroid receptor co-activators may be a mechanism for adapting to low levels of androgens after androgen deprivation therapy (Eder *et al.* 2001; Taplin *et al.* 2001). In human breast cancer, a nuclear receptor co-activator, AIB-1 (Amplified In Breast cancer-1) was

found to be amplified in approximately 5-10% of tumours (and also in 7% of ovarian cancers) with high associated expression in two-thirds of tumours analysed (Anzick *et al.* 1997). AIB-1 is the third identified member of the steroid receptor co-activator (SRC) family and was cloned as a direct result of searching for genes whose expression and copy number were elevated.

Recently, the first evidence of a role in prostate cancer for nuclear AR co-activators was reported (Gregory *et al.* 2001). The authors proposed a new mechanism for AR-mediated prostate cancer recurrence following androgen deprivation, based on overexpression of nuclear AR co-activators. The role of the AR together with 3 nuclear co-activators, TIF2 (transcription initiation factor-2), SRC1 and AIB-1, was investigated by comparing expression levels of each in samples of BPH, androgen-dependent and androgen-independent prostate cancer using immunohistochemistry and immunoblotting. The nuclear co-activators TIF2 and SRC1 were both found to show more intense immunostaining in hormone-refractory tumours compared with BPH or androgen-dependent tumours. In contrast, no immunostaining for AIB-1 was detected in any of the 3 specimen types, suggesting no role in prostate cancer. Immunoblot studies confirmed overexpression of TIF2 in six of eight recurrent tumours, while barely detectable levels could be found in BPH and androgen-dependent tumours. Associated with these changes, were high levels of AR expression in the hormone-refractory tumours suggesting a possible relationship between the two. In the nude mouse CWR22 human prostate xenograft model, levels of TIF2 and SRC1 were shown to decrease following castration, only to rise coincidentally with the emergence of recurrent prostate cell proliferation and an

androgen-insensitive phenotype, providing further evidence for a role for nuclear co-factors in the development of androgen-resistance.

Using two cells lines with a mutant AR (LNCaP T877A and CWR22 H874Y), which have broader ligand specificity than wild type AR, Gregory *et al.* also demonstrated by over expressing TIF2, the adrenal androgens (androstenedione and dihydroepiandrosterone) could activate transcription by the DNA and ligand binding AR507-919 receptor fragment. Such extended ligand specificity in the receptor, caused by these AR mutations, could enhance AR binding of adrenal androgens when androgen deprivation and co-activator overexpression occurs.

As increasing number of steroid receptor co-activators are being discovered, it seems likely considerable further information on the role of these proteins in prostate cancer will become apparent. Co-repressors of the AR may also play a role in prostate cancer but investigations are more limited. When AR is bound to an anti-androgen, the loss of co-repressor proteins might result in activation of the receptor. This has not been demonstrated to date in the AR but has been suggested for the ER and PR receptors (Lavinsky *et al.* 1998).

Mutations in Heat Shock Proteins

Mutations in heat shock proteins (HSP), that chaperone the AR in the unliganded state, may also contribute to HRPC. HSP 70 has been shown to complex with AR

and DNA and a mutated form of the protein HSP 70 can activate AR (Taplin *et al.* 2001).

Stabilization of the androgen receptor

Gregory and colleagues have reported that stabilization of the AR in the recurrent prostate cancer cell line CWR22 is associated with hypersensitivity to low levels of androgens. This phenomenon resulted in a four-fold decrease in the concentration of DHT required to promote tumour growth (Gregory *et al.* 2001). Similar findings were not seen in the androgen-dependent LNCaP cell line under the same conditions.

Increased Local Bioavailability of Androgens

Relapse during androgen withdrawal therapy has been suggested to be mediated in part by local androgen-dependent mechanisms (Labrie *et al.* 1993). The conversion of adrenal androgen precursors to dihydrotestosterone via testosterone in local tissues is a recognised process (Labrie *et al.* 1993). While serum levels of testosterone fall by 95% following castration, in prostate tumours, several studies have shown that dihydrotestosterone levels are decreased by only approximately two thirds (Labrie *et al.* 1993). It is therefore possible that after androgen deprivation therapy, a compensatory increase in the conversion of adrenal precursor steroids to active dihydrotestosterone occurs within the tumour. Such local androgen production would allow androgen-dependent tumour survival and growth at apparently castrate serum testosterone levels.

Local biosynthesis within prostatic tumours would suggest that maximum androgen blockade therapy may partially block local androgen production. The failure of such an approach to favourably influence survival in patients (Prostate Cancer Trialists' Collaborative Group 2000) argues strongly against this being a significant mechanism for progression to hormone-refractory prostate cancer, and this mechanism is now not widely regarded as contributing significantly to progression of disease.

Androgen Gene Receptor Amplification in Prostate Cancer

Development and progression to HRPC is believed to be determined by multiple genetic aberrations, the sequence and exact nature of which remain incompletely understood (Bubendorf *et al.* 1999). Amplification of genetic material has previously been shown to mediate resistance to therapy (e.g. methotrexate resistance due to amplification of the dihydrofolate reductase gene, Nowell 1986). Amplification of a gene is defined as an increased gene copy number in comparison to a reference gene, used to mark the normal gene copy number. Gene amplifications have been reported less frequently in prostate cancer than in most other carcinomas (Bubendorf *et al.* 1999).

In the early 1990s, increased gene copy number in the Xq11-q13 region was demonstrated in a small series of HRPC cases (4/9, 44%), using the genome-wide screening technique, comparative genomic hybridization (CGH, Kallioniemi *et al.* 1992; Visakorpi *et al.* 1995). The androgen receptor gene had previously been

reported as mapping to this area several years earlier (Brown *et al.* 1989; Kuiper *et al.* 1989) and the gene appeared a likely candidate for the amplified material. Initial Southern and slot blot analysis on the samples confirmed this, showing an approximate four-fold amplification of AR in recurrent tumours. In one patient where a corresponding primary tumour was also analysed, no evidence of amplification was seen (Visakorpi *et al.* 1995). Confirmation of these preliminary findings was performed using a larger numbers of patients, and required the use of formalin-fixed, paraffin-embedded archival tissue. Interphase fluorescence *in situ* hybridization (FISH) was used to determine AR gene copy number in these specimens (Hyytinen *et al.* 1994).

In the initial publication by Visakorpi *et al.*, the AR gene probe used in the FISH was found to map to the precise chromosomal location where gene amplification was detected by CGH (Visakorpi *et al.* 1995). FISH analysis of the same paired sample as the Southern and slot blot analysis confirmed a five-fold amplification in the AR gene relative to the X chromosome. Altogether in this study, 23 HRPC cases were examined by FISH with 7 (30%) showing evidence of AR amplification. A high degree of variation within the tumour in the AR copy number was found, with up to 40 AR gene copies per cell seen, and a mean AR copy number ranging from between 3.8 to 21.5 per cell. FISH was also able to simply distinguish between tumours with AR gene amplification and those with gains of the entire X chromosome. Gain of all X chromosome material was detected as an increased copy number of both the AR gene probe and the reference probe for the centromeric X region. A further 8/20 (35%) of recurrent tumours showed this finding in addition to the 30 % AR gene amplification. All other clinicopathological characteristics of tumours that exhibited

AR gene amplification did not differ from those that did not show amplification. Also significantly, no evidence of AR gene amplification was seen in any of 16 untreated paired tumour samples examined. This provided strong evidence that AR gene amplification was selected for in the development of hormone-refractory disease and was implicated in disease progression. These observations also provided the first documented *in vivo* example of a gene amplification causing treatment failure in patients with any type of solid tumours, and was the first time gene amplification had been shown to be a cause of endocrine-therapy resistance in tumours.

The reported mechanism by which the AR gene amplification might lead to a growth advantage for HRPC suggested that elevated AR copy number facilitates tumour growth in the presence of the low serum androgen concentrations (found after conventional endocrine manipulation). The androgen receptor is known to be the key mediator of androgen-dependent cell growth in prostate cancer, and amplification of the gene for the protein may lead to an increased cellular ability to proliferate in the reduced concentrations of non-testicular androgens. Such tumour cells may have become 'hypersensitive' to androgens and this hypersensitization may be a further mechanism, apart from the development of hormone-independent clones, for tumour progression to HRPC.

Additional evidence has also emerged to suggest that a growth advantage associated with AR gene amplification may be occurring. Kaltz-Wittmer *et al.* have recently reported that tumours that exhibit AR gene amplification show a correlation with a

higher proliferation rate as measured by Ki67/ MIB1 index (Kaltz-Wittmer *et al.* 2000), suggesting that AR gene amplification and a growth advantage may be linked.

One of the main clinical implications of AR amplification as a mechanism for the development of HRPC is that failure of anti-androgen therapy is likely to remain mediated through androgen-dependent mechanisms. Such tumours appear to remain highly dependent on the low levels of circulating androgens after surgical or medical castration. Another clinical implication is that if HRPC tumours have AR amplification, they may respond more effectively to 'second-line' maximum androgen blockade (MAB, the addition of steroid or non-steroidal anti-androgen therapy to surgical or medical castration). Preliminary evidence from one report suggests that this might occur (Palmberg *et al.* 2000). Such a finding may also provide a rationale approach for selection of patients for second line MAB. If AR gene amplification occurs at first hormone relapse after monotherapy (surgical or medical castration), then more effective androgen depletion with MAB may lead to lower recurrence rates through lower rates of development of AR amplification. Limited *in vitro* evidence has already suggested that a rapid decline in the concentration of an essential growth agent may prevent amplification and resistance developing in tumours (Sausville 1993).

Since the original report of AR gene amplification in HRPC, a number of other studies have confirmed the findings (Koivisto *et al.* 1995; Koivisto *et al.* 1997; Bubendorf *et al.* 1999; Kaltz-Wittmer *et al.* 2000; Miyoshi *et al.* 2000; Palmberg *et al.* 2000; Edwards *et al.* 2001; Linja *et al.* 2001) (see **Table 1.2**). AR gene amplification has also been seen in patients treated with anti-androgen monotherapy

such as bicalutamide (Palmberg *et al.* 2000) as well as from the result of surgical or medical castration or MAB treatment. In only one study (Haapala *et al.* 2001), where the presence of AR gene amplification has been assessed by FISH, has no evidence of amplification been found (0/11 patients with local tumour recurrence after orchidectomy and bicalutamide). In addition to general confirmatory evidence for AR gene amplification in HRPC, these studies have also provided confirmation that AR gene amplification is a rare event in prostate carcinomas not previously exposed to any type of androgen deprivation. The only exception to this has been an abstract report by Ware *et al.* showing a higher rate of AR gene amplification in a small number (8/17) of primary cancers (Ware *et al.* 2000).

Table 1.2. AR gene amplification rates in HRPC and primary tumours.

First Author	Year	Tissue preparation used	AR gene amplification rate in HRPC (number of patients)	AR gene amplification rate in primary tumours (number of patients)
Visakorpi	1995	Nuclei	30% (7/23)	0% (0/16)
Koivisto	1995	Nuclei	30% (3/10)	0% (0/10)
Koivisto	1997	Nuclei	28% (15/54)	0% (0/26)
Bubendorf	1999	Section	23% (116)	1% (2/205)
Kaltz-Wittmer	2000	Nuclei	36% (22/66)	0% (0/22)
Myoshi	2000	Nuclei	20% (1/5)	0% (0/37)
Palmberg	2000	Nuclei	13% (10/77)	-
Ware	2000	Section	-	47% (8/17)
Linja	2001	Section	31% (4/13)	0% (0/33)
Edwards	2001	Section	15% (3/20)	5% (1/20)
Haapala	2001	Section	0% (0/11)	-

Legend: AR; androgen receptor, HRPC; hormone-refractory prostate cancer, Nuclei; isolated cell nuclei, section: tissue section mounts

Reported AR gene amplification rates in all of these papers have been approximately 15-30% and are generally quite consistent.

More recently, AR gene amplification has been shown to be directly associated with overexpression of the AR (demonstrated by elevated mRNA levels, Linja *et al.* 2001). Using real-time reverse-transcription polymerase chain reaction, Linja *et al.* showed that all of the hormone-refractory tumours they examined expressed AR,

with an average 6-fold higher expression than androgen-dependent tumours (Linja *et al.* 2001). In 4 /13 HRPC tumours, AR gene amplification was also seen, with these tumours having an average of 2-fold higher level of AR expression than tumours without AR gene amplification. In addition, a tumour model system containing the AR gene amplification and high levels of expression of AR was developed. This should allow further studies of the functional significance of AR gene amplification.

Functional cellular consequences of AR gene amplification have already been detected. Koivisto and Helin correlated PSA expression by using a modified histoscore method with AR gene amplification in 24 HRPC cases (Koivisto *et al.* 1999). PSA immunostaining in AR gene-amplified tumours was twice as high as non-amplified tumours ($p=0.054$). AR gene copy number was also positively correlated with PSA modified histoscore in the gene-amplified tumours. The authors concluded that AR gene amplification leads to upregulation of the PSA gene.

The studies of AR gene amplification to date have mainly described locally recurrent prostatic disease, with only two studies including soft tissue metastases (Bubendorf *et al.* 1999; Kaltz-Wittmer *et al.* 2000). Studies of metastases are of importance because these sites are primarily responsible for clinical outcome in patients (Bubendorf *et al.* 1999). These sites are also the primary targets of local and systemic therapy. It is possible that local tumour progression and progression at metastatic sites represent two different phenomena and that tumour in the prostate may not accurately represent the tumour clone or clones causing disease progression (Palmberg *et al.* 2000). No studies have yet reported AR gene amplification rates

from HRPC bone metastases. As bone metastases are the commonest site of HRPC, this was the primary rationale for the investigation that forms this thesis.

1.5.4 Other Chromosomal Changes in Prostate Cancer (see Fig 1.3)

Screening of primary and recurrent prostate cancers for genetic alterations is required for better understanding of the development and progression of the disease. Classical cytogenetics has produced relatively little data on common solid tumours in comparison with the large amounts of data on haematological malignancies (Kallioniemi *et al.* 1994). The introduction of localised fluorescence in situ hybridization techniques (FISH) in the 1980s resulted in greatly improved knowledge about specific genetic abnormalities within solid tumours. A further modification of FISH, Comparative Genomic Hybridization (CGH) was developed in the early 1990s to assess genome-wide changes rather than alterations at specific loci (Kallioniemi *et al.* 1992). Moderate to large size gains and losses of genetic material in tumours can be screened for by this method, and CGH has resulted in the detection of a wide range of abnormalities, further discussed below.

Other Amplifications and Gains in Genetic Material in Prostate Cancer

Amplification of the AR gene is not the only type of gene amplification seen within prostate cancers. Amplifications are already known to be more commonly found in recurrent tumours, in contrast to primary tumours (where such changes are seldom seen, Koivisto 1996). Other gene amplifications discovered within recurrent prostate

tumours include the Elongin C gene (Porkka et al. 2002), the EIF3S3 gene (Saramaki et al. 2001), the urokinase gene (Helenius et al. 2001) and the hypoxia-inducible factor 1alpha gene (Saramaki et al. 2001). The significance of these findings in relation to recurrence and progression to hormone-refractory disease is not yet understood.

In addition to single gene amplifications, CGH studies have also aided the detection of more widespread gains of genetic material within prostate cancers (for reviews see Karan *et al.* 2003, Nupponnen and Visakorpi 2000 & 1999, Bova and Isaacs 1996 and Koivisto 1996). The commonest site for a gain of genetic material within prostate cancer is chromosome 8q, found to occur in over 50% of cases in all studies (Karan *et al.* 2003). Putative genes of interest in this area include the MYC oncogene at 8q24 and the EIF3S3 gene. To date, gains of chromosomal material at 1q, 2p 3p, 3q, 4q, 7p, 7q, 8q, 11p, 11q, 12q, 18q and Xq have all been described (Karan *et al.* 2003, Nupponnen and Visakorpi 2000 & 1999, Bova and Isaacs 1996 and Koivisto 1996). In most cases, less than 25% of tumours analysed have shown such gains. The implications of genetic gains within such tumour are not clear, apart from possible oncogene amplifications. Very large amplifications, such as that of c-erbB-2 in breast cancer, have not yet been identified in prostate cancer.

Losses of Genetic Material in Prostate Cancer

The loss of gene sequences has been associated with loss of tumour suppressor genes (TSGs) in a number of cancers (Bova and Isaacs 1996). A search for critical TSGs in

prostate cancer may be aided by identifying losses within regions of genetic material by CGH. It is not yet known how often such losses of genetic material within tumours actually represent changes that are important for tumour survival.

Losses of genetic material are more common in early prostate cancers. Koivisto has estimated that losses and deletions of genetic material are five times more common than gains and amplifications within primary prostate tumours (Koivisto 1996). In a similar way, recurrent tumours are known to have approximately four times more genetic alterations than primary tumours (as detected by CGH, Nupponen and Visakorpi 1999). These findings suggests that the early development of prostate cancers may be attributable to inactivation of TSGs, and that later progression is associated with gain of function.

Areas of loss of chromosomal material already identified by CGH with prostate cancers include 1p, 2q, 5q, 6q, 7p, 7q, 8p, 9p, 10p, 10q, 13q, 16q, 17p, 17q, 18q, 19p, 19q, 20q and 22q. In only two of these areas (8p and 13q) are losses particularly common (greater than 50% of all cases). The tumour suppressor retinoblastoma gene (RB) is known to be located at 13q14 but in all cases including this, the full significance of such losses is still unclear.

There are no reported CGH studies in bone metastases from hormone-refractory prostate cancer patients in the literature. The technique may prove practical for screening bone metastases from HRPC patients for genetic alterations. However, it has already been noted that CGH has been under used in primary bone marrow malignancies that contain a larger population of tumour cells (Heller *et al.* 2000).

This may be because bone marrow aspirates are too limited to perform DNA-extraction upon or because trephine samples are more difficult to work with (Heller *et al.* 2000, Alers *et al.* 1999).

1.5.5 INVESTIGATION AIMS, RATIONAL AND HYPOTHESES:

AIM: To identify genetic changes associated with the development of hormone resistance in bone metastases from men with prostate cancer.

The specific objectives were to:

1. Identify a method for obtaining cancer-containing bone biopsies routinely from men with HRPC, and compare cell yields in aspirate and trephine biopsies.
2. Collect a bank of bone and bone marrow biopsies from patients with HRPC, including matching primary tumour samples where available.
3. Develop a means of processing bone biopsies that retains the integrity of DNA and protein for FISH and immunohistochemical analyses.
4. Measure the prevalence and level of AR gene amplification in bone metastases from HRPC and their matching primary tumours.
5. Compare immunohistochemistry with FISH as a means of detecting AR gene amplification in bone metastases from HRPC.
6. Identify genetic changes in HRPC

RATIONALE

Prostate cancer spreads in most men to bone. Initially, the metastatic disease usually responds to hormone therapy, but after a mean interval of 12-18 months, hormone-resistant disease develops and there is little further treatment available beyond palliative measures.

Hormone-resistant prostate cancer is difficult to study in the laboratory because it is not usually biopsied. In the rare instances where bone biopsies are available, the cancer cells are degraded for biochemical purposes (studies of protein and DNA) by the decalcification procedures. I set out to study hormone-resistant prostate cancer, with the ultimate goal of finding better treatments for this disease. However, before being able to study the disease, the technical issues of (1) obtaining bone metastases routinely and (2) extracting or processing the tissue without destroying protein and DNA had to be overcome. Therefore, the first two hypotheses I set out with were:

1. Cancer-containing bone biopsies can be obtained routinely from men with HRPC.
2. Bone biopsies can be processed without destroying the tissue for immunohistochemical and FISH analyses.

Having overcome these obstacles, it was then possible to proceed to use the biopsies I had obtained to study the molecular mechanisms by which prostate cancer become hormone resistant. I tested three further hypotheses:

3. HRPC is associated with AR gene amplification in bone biopsies.
4. AR gene amplification demonstrated by FISH can be substituted by AR gene amplification demonstrated using immunohistochemistry.
5. Additional genetic changes occur in HRPC that can be found by comparative genomic hybridisation.

The third hypothesis was proven by the first demonstration of AR gene amplification in bone biopsies. Immunohistochemistry did not appear to provide a reliable substitute for FISH for the measurement of AR gene amplification. The CGH analyses did not provide useful data because of technical limitations and time constraints.

Chapter Two:
The comparative values of bone marrow aspirate and
trephine for obtaining bone scan-targeted metastases from
hormone-refractory prostate cancer.

2.1 Introduction

Samples of metastatic prostate cancer to bone are difficult to obtain. Since the introduction of radionuclide bone scanning in the 1980s, diagnostic bone marrow aspiration and trephine biopsies are seldom done to assess the bone marrow in patients with prostate cancer (including those with hormone-refractory disease). A bone scan now provides an objective and less invasive assessment of skeletal involvement by metastases, but does not yield tissue samples (Varenhorst *et al.* 1983). In prostate cancer, aspiration and trephine biopsy are now usually used for assessment of marrow reserves due to tumour infiltration, or as a research tool for assessing new techniques that may detect early metastatic spread (Deguchi *et al.* 1997). These findings, together with the difficulty in working with bony samples containing a mixture of hard and soft tissues (Alers *et al.* 1999), have resulted in bone metastases from carcinoma of the prostate being relatively understudied compared with prostatic tissue obtained during therapeutic transurethral resection (TURP).

There is little information in the literature on how best to target bone metastases for biopsy. The proposed method in this study was to target known bone metastases suitable for aspiration and biopsy, found at recent, routine, diagnostic bone scans (done for assessment of suspected tumour progression, or new bone symptoms). This would ensure patients would not require further tests to identify bone lesions if they were agreeable to bone marrow aspiration and biopsy. A Medline search for information using this approach revealed no studies with an assessment of this methodology, effectiveness or tumour yield (Chua *et al.* 1969; Sy *et al.* 1973; Crisp

1976; Spiers *et al.* 1982). Previous experience in the laboratory where this work was performed, had also shown that when bone marrow samples were obtained, aspirates from hormone-refractory prostate cancer patients rarely contained cancer cells suitable for study (T. Begum, personal communication).

2.2 Methods

2.2.1 Patient Selection and Bone Scanning

The project was given approval by the joint University College London/University College London Hospitals Research Ethics Committee (Reference Number 96-3391). Over an eleven-month period, patients were sought from both inpatients and outpatients under the care of Dr SJ Harland and Dr HA Payne at the Middlesex Hospital. For all patients, the history and previous diagnostic planar Technetium-99^m hydroxymethylene diphosphonate (750 MegaBecquerels 99^mTc-HDP) bone scans (hereafter called bone scan) were first reviewed for the presence of bone metastases affecting the sacro-iliac region suitable for biopsy. All bone scans had been performed using the standard operating procedure of the Institute of Nuclear Medicine, University College London. Bone scans had to have been performed within one year for patients to be eligible for the study. Hormone-refractory prostate cancer was defined as disease progression requiring a change in therapy after at least one previous hormonal manipulation and associated with a rising serum PSA. Previous radiotherapy to the sacro-iliac region excluded the patient from the study because of possible confounding radiation-induced cytogenetic changes.

If prospective patients for the study were identified, they were approached and asked to participate. A full discussion of the aims of the study was undertaken, and patients were given written information material to take away to read (see Appendix Two). If the patient was agreeable to the study, written informed consent was obtained and stored within the hospital notes. Permission from the patient was also obtained to contact the Pathology Department of the hospital where any primary tumour biopsy had been performed, to obtain material for the study of matched pairs of samples before and after the development of HRPC.

In addition to the standard method of bone scans described above, three study patients were also approached to undergo whole body ¹⁸-Fluorine (200 MegaBecquerels, MBq) positron emission tomography bone scans (hereafter called ¹⁸F PET), ^{99m}Tc (375 MBq) nanocolloid bone marrow scans (hereafter called nanocolloid bone marrow scan) and MRI scans of the pelvis (including marrow images using short tau inversion recovery sequences, STIR). The aim of these scans was to either look at the pattern of disease in three dimensions or to look at changes in the bone marrow adjacent to known metastases. A specific ARSAC licence was obtained for these three patients. Plain x-rays of the pelvis were also performed on all patients. All imaging studies were completed prior to each patient undergoing bone marrow aspiration and biopsy.

2.2.2 Sample Collection and Processing

Comprehensive training in bone marrow aspiration and trephine biopsy was given by the haematology service at UCLH Trust prior to undertaking the collection of study cases. All samples were taken by myself, using local anaesthetic at a site selected with direct reference to the bone scan. The posterior superior iliac spine was located and the site of biopsy chosen with reference to this. Clinical tenderness to palpation was used to help identify metastatic sites if apparent. If the proposed area chosen was not able to be safely biopsied (inaccessible to the aspiration needle), then the posterior superior iliac spine was used instead.

Bone marrow aspirates were taken first, using a 15G MarrowgaugeTM needle (Rocket Medical, Watford, UK). Bone marrow trephine (s) were then taken with a Becton-Dickinson Bone Marrow Biopsy needle (Becton-Dickinson, New Jersey, USA). Haemostasis was achieved and analgesia given if required.

Diagnostic bone marrow aspirate slides were made using the standard smear technique and sent to the haematology service for reporting. Bone marrow trephines were immediately placed in formalin-saline and sent for histological assessment by the UCLH Trust Histopathology Department. Standard stains and immunohistochemistry (PSA, prostatic acid phosphatase and the cytokeratin markers CAM 5.2 and MNF116) were performed on all samples. After eight patients had undergone aspirate and trephine biopsies, half of the aspirate sample slides were also sent for immunocytochemistry for PSA and CAM 5.2.

For all study patients, serum PSA and the full blood counts (FBC) were recorded at the time of progression. A manual count of the number of visible hot spots on the bone scans was recorded.

2.3 Results

Twenty of the 29 patients approached agreed to participate (69%, see **Table 2.1** for clinical details). One patient had co-existent chronic myeloid leukaemia (CML). The mean age of patients was seventy-years-old. The number of previous treatments for prostate cancer ranged from one to six, covering most curative and palliative modalities used for prostate cancer treatment. Patients had undergone the treatments listed in **Table 2.1** prior to bone marrow aspiration and trephine biopsy. Previous therapy was usually systemic in nature and intended to provide palliation. Only 4 patients had originally been treated radically. The majority of patients (12/20) had presented with metastatic disease (M1).

Table 2.1. Clinical characteristics of bone marrow aspiration and trephine patients.

Patient Number	Age	Stage at first presentation*	Matching Primary Tumour	Previous treatments for prostate cancer
1	76	M1	N	O, S & HC
2	65	T1C	N	MAB, HC, S
3	70	T3 M1	N	MAB
4	70	M1	N	G, S & HC
5	78	M1	N	TUR & O, F, S & HC
6	78	M1	Y	TUR, MAB, S & HC, Mitox
7	69	M1	Y	O, CPA
8	75	pT3 N0	Y	RP, O, F, S & HC
9	70	M1	N	O, F, S, ECarboF, Mitox, 5-FU/FA
10	74	M1	Y	G, Zoled, F
11	65	pT3b	Y	RP, G & CPA, B
12	72	M1	N	O, CPA, Z, S, B, Mitox
13	62	M1	N	MAB
14	60	M1	N	G, Estra, S & HC
15	75	M0	Y	O, TUR, Zoled, S & HC
16	73	M0	y	RT & G, S & HC, B
17	62	M1	N	G, B, P, ECarboF, S
18	64	T2 N0	N	RT, MAB, S & HC
19	66	M0	Y	TUR, G
20	88	M1	N	G

(See legend below)

Table 2.1 (continued). Clinical characteristics of bone marrow aspiration and trephine patients.

Patient Number	Number of hot spots on bone scan	PSA at time of biopsy	FBC at time of biopsy		
			Hb	WBC	Platelets
1	11	47	13.5	14.6	243
2	27	95	11.3	4.0	319
3	7	267	13.4	7.4	224
4	18	69	10.6	6.5	267
5	58	27	13.8	11.0	297
6	42	3064	11.6	5.1	225
7	21	276	11.5	7.2	205
8	53	19	13.6	9.4	264
9	31	398	12.2	10.4	286
10	32	45	15.9	9.8	114
11	8	29	14.0	9.7	209
12	18	3306	12.7	8.8	179
13	Superscan	311	10.5	10.5	391
14	42	111	8.3	3.6	277
15	38	182	12.2	9.3	487
16	47	120	11.5	9.9	269
17	73	1730	11.5	7.6	310
18	53	41	12.4	6.1	400
19	11	375	10.0	2.6	234
20	29	133	9.8	2.9	174

* 1997 UICC T N M Stage

Legend for Table 2.1

B; bicalutamide, CPA; cyproterone acetate, Estra; estramustine, ECarboF: epirubicin, carboplatin & 5-FU, F; flutamide, 5-FU/FA; 5-fluorouracil & folinic acid, G; Goserelin, HC; hydrocortisone, MAB: maximum androgen blockade, Mitox; mitoxantrone, N; no, O; orchidectomy, P; prednisolone, S; stilboestrol, RP; radical prostatectomy, RT; radical radiotherapy, TUR; transurethral resection, Y; yes, Zoled: Zoledronic acid.

All patients had elevated PSAs with a median of 126.5 ng/ml and a range of 19 to 3,306 ng/ml. Anaemia was a common finding on the full blood count (FBC) with 14/20 patients having haemoglobin below the laboratory reference range of 13 g/dl (see **Table 2.1**). However, only 3/20 patients had a white cell count (WBC) below $4 \times 10^9/l$ and only a single patient had an abnormally low platelet count (patient 10, $114 \times 10^9/l$). Two patients with a low WBC also had a low haemoglobin level suggesting possible marrow failure, but in both of these cases the bone marrow aspirates were negative and the trephine biopsies showed less than a 5% tumour cell infiltrate.

The range of lesions visible on the AP planar bone scans for the 20 patients ranged from 7 to over 50. In addition, one patient had a 'superscan' (widespread tracer uptake with no renal tracer excretion visible at the time of scanning). The two patients with blood counts suggestive of some marrow failure did not have very high hot spot counts on the bone scans. There was no correlation between the level of serum PSA and the number of hot spots on the bone scan ($r=0.12$, Spearman log rank test).

The results of the aspiration and biopsies are shown in **Table 2.2**. **Figure 2.1** shows the appearance of a typical marrow trephine sample as demonstrated by haematoxylin and eosin stain. **Figure 2.2** shows a high power photomicrograph of a further marrow trephine, demonstrating areas of tumour infiltration shown by PSA immunohistochemistry.

Table 2.2 Results of bone marrow aspiration and trephine biopsies.

Patient Number	Bone Marrow Aspirate	Bone Marrow Trephine
1	Negative, normal lineages	Positive, multiple tumour foci
2	Normal lineages, ? occasional suspicious cells	Negative
3	Negative, normal lineages	Positive (10% of marrow space)
4	Dry Tap	Failed
5	Negative, normal lineages	Negative
6	Dry Tap	Positive, Diffuse infiltration
7	Negative, haemodilute	Positive, 1 focus
8 *	Negative, normal lineages	Positive, 1 focus
9 * *	Negative, normal lineages	Negative
10 * *	Negative, normal lineages	Positive, multiple tumour foci
11 *	Negative, haemodilute	Positive, multiple tumour foci, known CML
12 *	Dry Tap	Positive, Diffuse tumour infiltration
13 *	Dry Tap	Failed
14 *	Dry Tap	Positive, Diffuse tumour infiltration

(continued over)

Table 2.2 Results of bone marrow aspiration and trephine biopsies (continued).

Patient Number	Bone Marrow Aspirate	Bone Marrow Trephine
15 *	Dry Tap	Positive diffuse tumour infiltration
16 *	Negative, normal lineages	Positive, multiple tumour foci
17 *	Negative, erythroid dysplasia	Positive, multiple tumour foci
18 *	Negative, normal lineages	Positive, multiple tumour foci (25% of marrow space)
19 *	Haemodilute, ? occasional suspicious cells	Positive, multiple tumour foci (< 5% of marrow space)
20 *	Negative, haemodilute	Positive, multiple tumour foci (< 5% of marrow space)

Legend for **Table 2.2**:

CML; chronic myeloid leukaemia.

* For the last eleven patients bone marrow aspirate slides (if obtained) were also sent for immunocytochemistry; in no instances did they show evidence of tumour cells.

† The 3 patients who underwent multiple bone imaging for assessment

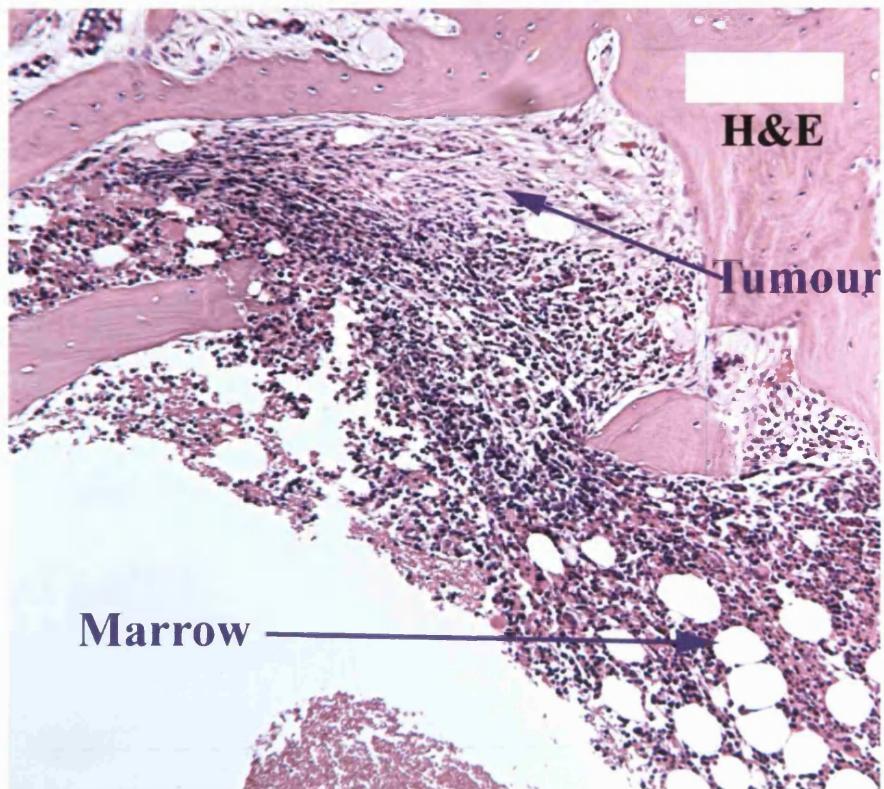


Figure 2.1

Low power haematoxylin and eosin section, showing tumour cells adjacent to decalcified trabecular bone (in pink) merging into normal bone marrow constituents ((x 100 magnification).

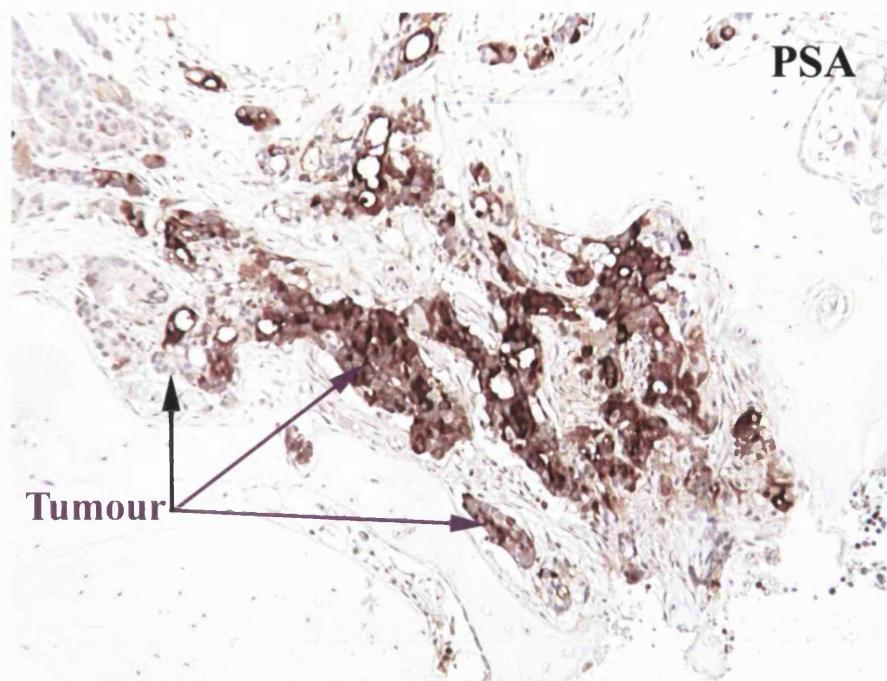


Figure 2.2

High power photomicrograph showing immunohistochemistry for PSA on a bone marrow trephine section. PSA on prostate tumour cells detected as brown membrane bound staining. Note some attempt at gland formation indicated by the arrow at the 12 o'clock position (x 400 magnification).

In two cases no material was obtained due to a dry tap on aspiration and a failed trephine biopsy, secondary to sclerotic bone (unable to be penetrated by the trephine needle). In the remaining eighteen cases, a suitable bone marrow trephine was obtained. For three of the eighteen trephine biopsies (17%) a normal marrow sample with no evidence of tumour deposits was obtained. Fifteen of the eighteen successful trephines (83%) contained metastatic tumour. In one of these samples only a single microscopic focus of disease was seen on immunohistochemistry, too small for further study.

Of eighteen attempts at bone marrow aspiration, no material in six was aspirated due to a dry tap. These failures were associated with either diffuse tumour infiltration in the trephine biopsy (4/6) or also had an unsuccessful trephine biopsy (2/6). Ten of the aspirates contained sufficient particulate material to assess. In one case suspicious cells were seen, but this was associated with a normal trephine biopsy. In the remaining cases no evidence of tumour cells was seen. Four aspirate samples were haemodilute and sub optimal for assessment. One of these was reported as containing possible occasional suspicious cells but the trephine showed low levels of tumour infiltrate (< 5%).

Of nine cases where aspirate slides were also sent for immunocytochemistry (PSA and CAM 5.2), no evidence of tumour infiltrates was seen, in full agreement with the haematological assessment.

The bone marrow sampling was generally well tolerated by patients with no major complications. Lignocaine 2% local anaesthetic was used for all patients. One patient

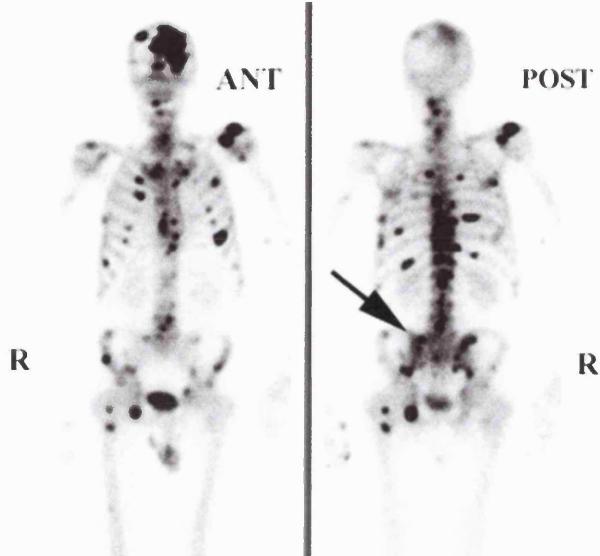
had a pre-syncopal episode that settled with a short period of bed rest after the procedure. The majority of patients were already on compound analgesics, non-steroidal anti-inflammatory drugs or opiates because of bone metastases prior to the aspirate and trephine biopsies. Local pain after the procedure was uncommon and patients seldom required extra analgesia. In three out of the twenty cases the patient would have declined to undergo a similar procedure again, two of these cases were in patients with a failed aspirate and trephine due to sclerotic bone.

For the three patients who underwent the other types of bone imaging (plain x-ray, ¹⁸F PET bone scan, nanocolloid bone marrow scans and MRI scans of the pelvis), the target lesion was successfully identified in all three patients on the PET bone scans and MRI scans of the pelvis, as shown in **Figure 2.3B and C**. **Figure 2.3A** shows the corresponding AP planar bone scan for this patient.

In no case did the nanocolloid bone marrow scan identify the target lesions in the sacro-iliac region (see **Figure 2.4A**). In only one patient did the nanocolloid bone marrow scan identify any bone marrow abnormality (a solitary accumulation defect which matched a known hot spot on the diagnostic bone scan, data not shown). Of the three plain x-rays taken, an abnormality at the site of the target lesion was seen in one case (data not shown). The corresponding nanocolloid bone marrow scan and pelvic x-ray for the patient in **Figure 2.3 A, B, C** are shown in **Figure 2.4 A and B**.

Figure 2.3

A



B



C

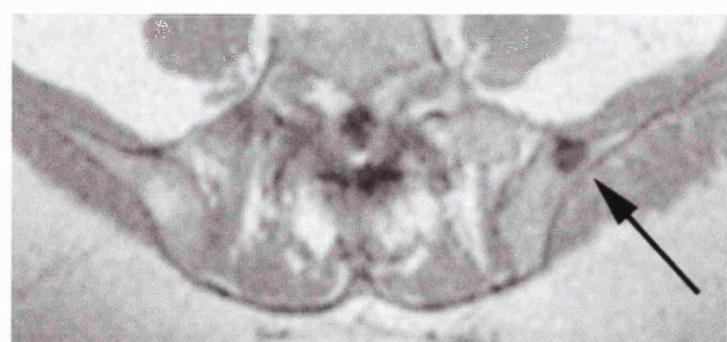


Figure 2.3

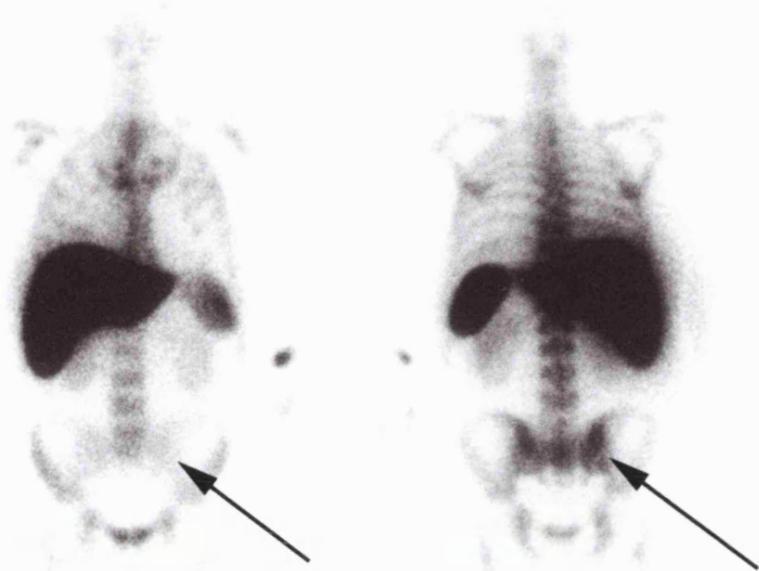
Bone scan (A), PET bone scan (B) and pelvic MRI (C) of patient 8 in whom we were unable to obtain useful tissue for molecular biology studies. **A.** 99m Tc-bone scan with targeted lesion shown by the arrow. **B.** 18F PET bone scans (axial view) of the pelvis showing the position of the targeted lesion. **C.** Axial pelvic MRI view of the target lesion.

Figure 2.4

ANT

POST

A



B

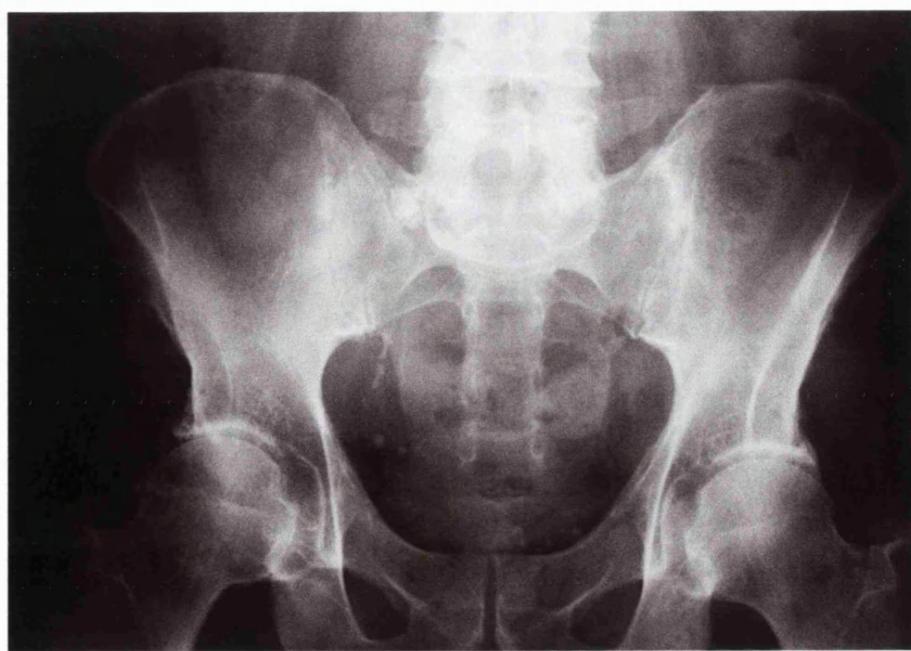


Figure 2.4

Further images from the same patient as **Figure 2.3. A.** ^{99m}Tc -nanocolloid bone marrow scan showing no evidence of an accumulation defect in the area of the sacroiliac region (or other sites). **B.** Plain x-ray of the pelvis showing no definite abnormalities at the site of the lesion visible on bone scan, PET bone scan or MRI.

2.4 Discussion

The detection of metastases within bone marrow by needle aspiration was first reported in 1936 and included a patient with metastatic carcinoma of the prostate (Rohr *et al.* 1936). Specific studies on the bone marrow changes in prostate cancer then began to appear in the 1940s and 1950s (Alyea *et al.* 1949; Clifton *et al.* 1952). Early studies were mainly in the context of diagnosis and staging, particularly with reference to diagnosing occult bone metastases radiologically, and to try and prevent unnecessary radical surgery (Welch *et al.* 1964). As new diagnostic techniques became available, such as improvements in chemical pathology (e.g. bone marrow acid phosphatase, Sy *et al.* 1973) histopathology techniques (e.g. immunohistochemistry, Mansi *et al.* 1988) and molecular biology (e.g. polymerase chain reaction, Wood Jr. *et al.* 1994) further studies have continued to be published.

Bone and bone marrow samples are inherently more difficult to work with than some other tissue types, due to the mixture of hard and soft material and the need for acid or EDTA decalcification. Tissue such as prostate gland, removed at transurethral resection, is justified on diagnostic and therapeutic grounds, as well as providing material for study. Bone marrow aspiration and trephine biopsy is no longer a standard diagnostic procedure in prostate cancer. Varenhorst *et al.* showed that bone scanning was superior to random bone marrow aspiration in staging prostate cancer in 1983 (Varenhorst *et al.* 1983).

Bone scans are the most sensitive bone imaging technique our patients routinely undergo, and bone marrow aspiration and trephine can be done by a trained individual without the aid of interventional radiology. The combined use of bone marrow aspiration and trephines that had been targeted by previously performed bone scans, appeared to be a good approach for obtaining cancer cells for research studies, but no published information on the success rates in prostate cancer bone metastases was available.

This method yielded a high sampling rate for trephine biopsies (15 / 20 attempted cases, 15 / 18 submitted trephines positive), but no yield for aspiration. It is known that trephine biopsy is more sensitive than bone marrow aspiration for the detection of metastatic disease in the bone and bone marrow (Bearden *et al.* 1974). However, there is considerable variation within the reported literature on the positive aspiration rate for bone marrow aspiration. The older literature on prostate cancer bone metastases reports positive aspiration rates between seven and fifty percent for mixed groups of early and advanced prostate cancer cases (although the majority of positive aspirates were in patients with known bone metastases in all studies, Alyea *et al.* 1949; Clifton *et al.* 1952; Welch *et al.* 1964; Mehan *et al.* 1966; Nelson *et al.* 1973). Early studies did not have the benefit of modern techniques such as immunocytochemistry. The use of immunocytochemistry did not increase the positive marrow aspiration rate in smears of the nine cases assessed here. In micrometastases from breast cancer, immunocytochemical methods can detect tumour cells in experimental marrow/tumour mixtures down to 0.00025% (Molino *et al.* 1991). Positive bone marrow aspiration rates using morphology assessment in patients with known bone metastases from breast cancer show a significant

proportion of tumour cells in the aspirate (40%, Ingle *et al.* 1978). In prostate cancer patients with known M1 disease, positive aspiration rates varied between 20-58% when random biopsies were performed in the older literature (Alyea *et al.* 1949; Clifton *et al.* 1952; Mehan *et al.* 1966; Nelson *et al.* 1973). Mansi *et al.* reported moderate to large numbers of cells in the bone marrow aspirate of eleven out of fifteen patients (73%) with known bone metastases, using a panel of immunohistochemical markers (Mansi *et al.* 1988). A previous preliminary study at our centre of prostate cancer patients with positive bone scans showed three of nine aspirates taken by the haematologist contained prostate cancer cells, and only one sample contained cells in large numbers (T Begum, personal communication).

It is difficult to explain the low aspiration rate here in comparison to other studies. Tumour load, as indirectly assessed by the combination of serum PSA (Grossmann *et al.* 2001), bone scan lesion count (Soloway *et al.* 1988) and FBC results, does not reveal results different to many other patients seen in the clinic with progressive prostate cancer. Reverse transcriptase polymerase chain reaction was not performed on any of the blood samples obtained, and therefore there is no available data on circulating tumour load in a more quantitative fashion.

One reason for lower marrow aspiration rates in bone metastases from prostate cancer (and other tumours) has long been recognised (Alyea *et al.* 1949). The failure to obtain any material on bone marrow aspiration is usually associated with a trephine biopsy that showed a significant tumour infiltrate and a marked fibrotic reaction within the marrow space. This occurred in 5 of the samples.

Positive random bone marrow trephine biopsy rates have been previously reported as being positive in five out of fourteen cases (36%) by Crisp and five out of six cases (83%) by Spiers (Chua *et al.* 1969; Sy *et al.* 1973; Crisp 1976; Spiers *et al.* 1982). In the biggest series reported by Sy *et al.*, thirty-two out of forty-one patients (78%) with advanced prostate cancer had positive random iliac bone biopsies. In the only other study of prostate cancer patients reporting targeted biopsies, Chua *et al.* obtained eighteen out of nineteen (95%) positive trephine biopsies in a group of patients with known bone metastases targeted by x-ray guidance (as part of a mixed series of random and image-guided bone marrow biopsies, Chua *et al.* 1969).

One reason for the failure of bone marrow trephine biopsy to obtain metastases (apart from significant osteosclerosis) is shown in **Figures 2.3 B** and **C**. Without image-guided biopsies, small lesions (such as those demonstrated) may be difficult to target accurately by clinical sampling alone. The standard diagnostic bone scan used to guide all our biopsies were two-dimensional planar (anteroposterior) images. The absence of information on the depth of the targeted lesion may limit the successful sampling of smaller lesions. Such depth information can be obtained from bone scans by using a tomographic technique on a diagnostic gamma camera (producing images that are very similar to the axial PET view shown in **Figure 2.3 B**). These types of views are rarely used for diagnostic scans in prostate cancer patients where the information required usually concerns the question: are there bone metastases present? or, have known bone metastases changed over time?

Magnetic resonance imaging of the pelvis and PET bone scanning can also provide information on target lesion depth (with MRI able to be used to measure an accurate

depth, **Figure 2.3 C**). When there was only a small target lesion, the use of extra information on depth still did not allow a successful biopsy to be taken. These imaging modalities are not routinely available for use on all patients to identify the site of disease in three-dimensions. It is hard to see how the rate of positive biopsies could be increased further using clinical rather than direct radiologically guided biopsies to target small metastatic deposits using the method described.

Magnetic resonance imaging scans have been shown to be a very sensitive method of detecting skeletal metastases compared with conventional bone scintigraphy (Algra *et al.* 1991; Steinborn *et al.* 1999). Planar bone scintigraphy has been compared with ¹⁸F PET bone studies by Schirrmeyer *et al.* in forty-four patients with known prostate (20 / 44), lung or thyroid carcinoma (Schirrmeyer *et al.* 1999). The bone scans had a decreased sensitivity for detecting benign and malignant lesions in the bony pelvis compared with ¹⁸F PET bone scans. The three ¹⁸F PET and pelvic MR images in our study were, however, not performed for direct comparison of sensitivity or specificity for lesion detection, but rather for further localization of a target lesion.

The results of the nanocolloid bone marrow studies on the three patients agree with previously published data. Haddock *et al.* reported nanocolloid bone marrow scintigraphy to be less sensitive than conventional bone scans for the detection of skeletal metastases from carcinoma of the prostate (Haddock *et al.* 1989). Bone marrow scans usually reveal cold spots (photon deficient areas due to defects in tracer accumulation) if significant marrow infiltration is present. Hot spots have also

been reported due to metastatic disease, but neither metastases nor peripheral marrow expansion due to central marrow failure were seen in the three patients scanned.

In conclusion, it has been shown in this part of the study, that trephine biopsy can be successfully used to obtain cancer cells from the majority of HRPC patients using previous diagnostic bone scans as a method for targeting bone deposits. The addition of other types of imaging in 3 patients did not help to increase yield of tumour cells.

Chapter Three:

Results of Formic Acid Decalcification of Bone Marrow

Trephine Samples.

3.1 Introduction

Production of paraffin-section material from tissue samples that contain bone requires decalcification. Techniques such as acidic decalcification or EDTA chelation are suitable methods. Acid decalcification is generally quicker than EDTA chelation, but studies have suggested that it results in hydrolysis of DNA (Alers *et al.* 1999), impairing the ability to perform molecular studies. Samples in this study were processed into paraffin-embedded sections at the Department of Histopathology, University College London Hospitals NHS Trust. In this department, decalcification is routinely undertaken using formic acid solutions of varying strengths. Such decalcification is suitable for certain routine molecular studies such as immunohistochemistry, but fluorescence in situ hybridization (FISH) is not routinely performed within the department.

3.2 Methods

Acid Decalcification of Trehpne Bone Samples

All bone marrow trephine samples were routinely decalcified for 12-18 hours using a 5% formic acid solution (Becton Dickinson Laboratory Supplies) in distilled water. For larger, autopsy bone marrow samples, a 10% formic acid solution was used for a 7-10 day period with replenishment of the solution on one to two occasions during the decalcification period. All samples were then cut into 5-micron thickness sections and mounted on silane-coated slides in preparation for FISH. Adjacent sections were mounted on uncoated slides for staining with haematoxylin and eosin.

3.3 Results

Five percent formic acid decalcification resulted in successful FISH in all trephine samples analysed for AR gene copy number in bone metastases from HRPC (12/12 cases). In contrast, for the five larger autopsy bone marrow specimens, decalcification in 10% formic acid solution resulted in no successful FISH studies (see Chapter 4 for the results of the FISH investigations). Where there were soft tissue metastases from the same autopsy cases available for study (three out of five cases), FISH was successful in two out of three samples (the remaining two samples coming from patients with only metastatic bone disease at post-mortem).

3.4 Discussion

The literature on the best methods for decalcification of bone marrow samples for molecular studies is small. Routine acid decalcification of bone marrow trephine and autopsy bone samples has been reported to result in total failure to obtain DNA for *in situ* hybridization (ISH), comparative genomic hybridization (CGH) and flow cytometry studies in ante-mortem trephine and post-mortem bone marrow samples from prostate cancer, in a small study by Alers *et al*, 1999. In contrast, within this study, decalcification using a 10 % EDTA solution for three autopsy bone marrow specimens alone, resulted in successful ISH, CGH and flow cytometry and was also reported to give better preservation of architecture for routine haematoxylin and eosin staining and immunohistochemistry. The authors concluded on this basis, that EDTA was highly preferable to their routinely used acid decalcifier where studies on DNA were required.

Acid decalcification was also used for the specimens in the current study, but a different preparation was used (5-10% formic acid rather than RDO, a proprietary agent produced by Apex Engineering Products, Plainfield, IL, USA), as used by Alers *et al.*, 1999. The finding here suggest that brief decalcification of small samples in a 5% formic acid solution does not result in significant DNA acid hydrolysis.

There is little published information available on the effects of formic acid decalcification on DNA degradation. A Medline literature search for the years 1966-2002 was unable to find other reports of successful studies using FISH, from bone marrow trephine biopsies decalcified in formic acid. Sarsfield *et al.* reported that formic acid decalcification of bone marrow trephines degraded DNA for polymerase chain reaction (PCR) studies using specific PCR primers (Sarsfield *et al.* 2000). In this small comparative study of formic acid and EDTA decalcification from eleven bone marrow trephine specimens, similar quantitative amounts of DNA could be retrieved using both decalcification methods, but formic acid pre-treatment resulted in a degraded DNA smear and failure to obtain specific PCR products in the majority of samples tested.

Provan *et al.* have also investigated formic acid decalcification and were able to generate a 294 base pair (bp) DNA PCR product in only six out of ten samples following formic acid decalcification of paraffin-embedded bone marrow biopsies (Provan *et al.* 1992).

In contrast, EDTA decalcification resulted in successful amplification of specific DNA PCR products up to 643 bp in the study by Sarsfield *et al.* and the author's previous experience of EDTA for decalcification of bone marrow trephines alone strongly supports this finding (Wickham *et al.* 2000). None of the samples in the current study were decalcified using EDTA.

In conclusion, it has been shown that routine acid decalcification with 5% formic acid can satisfactorily preserve DNA for certain types of molecular biological studies (such as FISH) on prostate cancer bone metastases. Longer and stronger acid decalcification resulted in unsuccessful FISH in all autopsy specimens whilst non-decalcified material from the same post-mortem samples was shown to work satisfactorily, strongly suggesting it is the decalcification process that is the cause for the failure (as the remaining methodology for the FISH is identical for all types of samples).

Decalcification using EDTA solution has been reported to offer the best chance of successful DNA retrieval from bone tissue in two studies (Alers *et al.* 1999; Sarsfield *et al.* 2000) and appears to be the method of choice for decalcification in a prospective study of bone marrow trephine and autopsy samples. However, the current study has shown for the first time that limited 5% formic acid decalcification can be used for FISH studies in bone metastases from carcinoma of the prostate.

Chapter 4:

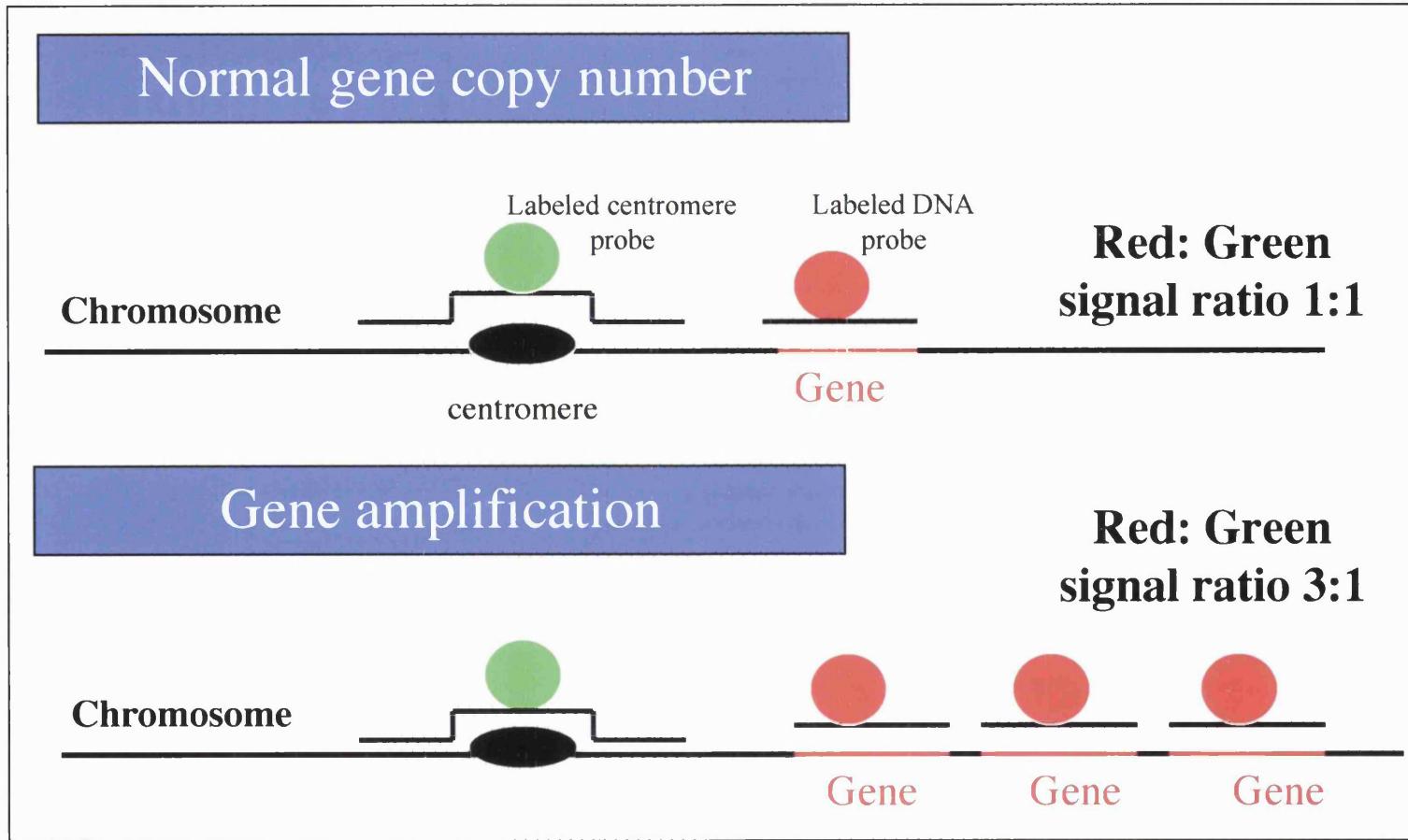
**Amplification of the androgen receptor gene in bone
metastases from hormone-refractory prostate cancer by
fluorescence in situ hybridization.**

4.1 Introduction.

In situ hybridization, the detection of nucleic acid sequences in their native place, was first reported in 1969 (Gall *et al.* 1969). Original techniques used radio-labelled complementary sequence probes, with nonisotopic in situ hybridization first described 6 years later in 1975, using an biotin-avidin binding method (Manning *et al.* 1975). It was the development of fluorescence in situ hybridization (FISH) methods by labelling a DNA probe with a fluorophore visible in ultraviolet light, that revolutionized cytogenetics, in the 1980s, bridging the gap between conventional and molecular genetics. The technique enables rapid detection of chromosome aberrations such as gene deletions, amplifications (see **Figure 4.1**), and translocations, in many tissue types (including tumours), using both fresh and archival material (for a review of the applications of FISH, see Gozzetti *et al.* 2000). These genomic abnormalities contribute to disease by altering the normal pattern of gene expression (King *et al.* 2000). Aneuploidy is well recognised to be a contributing cause of cancer and genetic disease.

The ability to analyse metaphase cells using FISH is highly advantageous as classically, such cells are difficult to analyse because of poor chromosome preparations and the inadequacies of conventional chromosome banding (Nath *et al.* 2000). Fluorescent DNA probe technology is also versatile. It can be applied to locus-specific genetic examination, as in this chapter, or used in related techniques such as comparative genomic hybridization to screen for genetic change across the whole genome (see Chapter 5).

Figure 4.1. Schematic representation of FISH, showing signal appearance in gene amplification and normal gene copy number



FISH like Northern and Southern blotting, relies on the Watson and Crick base pairing interaction between two polynucleotides annealing with complementary sequences. Unlike Northern or Southern blots, the target sequences are left within tissue, with no need to isolate nucleic acids prior to probing.

Detection of gene copy number change is reliable by FISH with high sensitivity and specificity. The sensitivity of the technique for nucleotide sequence detection is in the megabase (MB) range without computerised enhancement techniques (which can now reduce this down to less than 10 kilobases).

FISH may be applied to either formalin-fixed paraffin-section specimens or to whole isolated nuclei preparations. Both variations of the technique have advantages and disadvantages and have been used in the study of prostate cancer (see **Table 1.2**). In histologic section material, the key disadvantage is that many cells will exhibit aberrant *in situ* hybridization copy number due to the effects of nuclear slicing, making chromosome deletions more difficult to evaluate. Also in histologic sections, it is possible to overestimate gene amplification, due to poor discernment of overlapping nuclei (increasing the probe signal count of the nuclei assessed). Locus-specific *in situ* hybridization on histological sections, as used in this chapter, has the advantage of being able to study tumours in their native architecture. This can be essential in certain tumour types such as breast or prostate cancer (Fletcher 1999).

The alternative approach, using isolated, intact nuclei for FISH analysis is generally simpler to perform and avoids the problem of nuclear truncation. The loss of tissue

architecture can be a major problem and was the main reason why tissue section material was chosen for the study.

4.2 Methods.

4.2.1 Patients and Samples

The demographics for the patients in whom bone marrow trephine samples contained sufficient material for study was available, have been listed in Chapter Two (see **Table 2.1**). As well as these tumour samples, a number of other types of specimens were obtained during the study. Post-mortem tissue samples (as formalin-fixed, paraffin-section material) were obtained from patients with HRPC (as defined in the study criteria) that died during the study period. Consent for the study of this material was obtained from relatives of the deceased (see Appendix 1). The study was discussed with the pathologist performing the post-mortem prior to collection of material. Both bone and soft tissue metastases were both obtained if present at post-mortem.

Further patients under the care of Dr Harland and Dr Payne who underwent therapeutic TURP during the study period (because of HRPC and in whom bone scans had shown metastases that were not affecting the sacroiliac region), were approached for inclusion of the TURP samples into the study. Written informed consent was also obtained from these patients.

The clinical characteristics of the patients for whom sample material was obtained but not from bone marrow trephine are listed in **Table 4.1** and **Table 4.2**.

Table 4.1

Clinical characteristics of other study patients not undergoing bone marrow aspiration and trephine biopsy.

Patient Number	Age	Stage at first presentation *	Specimen Type	Matching Primary Tumour
21	64	T3 N0 M0	PM (BM, LN)	Y
22	66	T3 N0 M1	LN	N
23	64	M1	PM (BM)	Y
24	75	M1	PM (BM, LN)	Y
25	63	pT3b N1 M0	PM (BM, LN)	Y
26	61	M1	PM (BM)	N
27	71	pT3a N0 M0	TURP	Y
28	74	M1	TURP	N
29	69	M1	TURP	N

* 1997 UICC T N M Stage

Table 4.1 (continued).

Clinical characteristics of other study patients not undergoing bone marrow aspiration and trephine biopsy.

Patient Number	Previous treatments	PSA ant time of biopsy	FBC at time of		biopsy Platelets
			Hb.	WBC	
21	O, S, HC, ECarboF	600	7.5	5.5	58
22	O, F, S, HC, ECarboF, B, Mitox	1249	9.5	9.0	278
23	G, B, S, Carbo	193	8.6	26.7	150
24	G, S, HC	2110	9.1	4.5	169
25	RP, RT, L, F, ECarboF, S	54	9.3	5.1	134
26	C, G, S, HC	643	9.7	9.0	239
27	RP, G, F, S, ECarboF, HC	82	10.2	7.4	250
28	O, F, S, HC, B, TURP	96	11.5	7.9	237
29	G, B, S, HC, TURP	207	13.1	9.9	307

Legend:

B; bicalutamide, BM; bone marrow, Carbo; carboplatin, ECarboF: epirubicin, carboplatin & 5-FU, F; flutamide, G; goserelin, HC; hydrocortisone, L; leuprolide, LN; lymph node, Mitox; mitoxantrone, N; no, O; orchidectomy, PM; post mortem S; stilboestrol, RP; radical prostatectomy, RT; radical radiotherapy, TURP; transurethral resection of prostate, Y; yes.

Table 4. 2

Characteristics of other tissue samples not obtained by bone marrow aspiration and trephine biopsy.

Patient Number	Post-mortem bone sample	Other Tissue
21	Diffuse marrow infiltration	PM LN, replaced by tumour
22	-	LN, replaced by tumour
23	Diffuse marrow infiltration	-
24	Diffuse marrow infiltration	PM LN, replaced by tumour
25	Diffuse marrow infiltration	PM LN, replaced by tumour
26	Diffuse marrow infiltration	-
27	-	TURP, abundant tumour
28	-	TURP, abundant tumour
29	-	TURP, abundant tumour

Legend:

LN; lymph node, PM; post-mortem, TURP; transurethral resection of prostate.

4.2.2 FISH Methodology

The work was performed in the Department of Surgery Laboratories, Glasgow Royal Infirmary, under the supervision of Dr J. Edwards. The FISH protocols used for the soft tissue samples and bone samples are listed in **Appendix 2**. Briefly, for all soft tissue samples, the following steps were carried out using a VP2000 robotic slide processor (Vysis, UK Ltd). Five μm formalin-fixed, paraffin-embedded tissue sections, mounted on silane slides were dewaxed, rehydrated and treated with a tissue permeabilisation agent (0.2N HCl), followed by exposure to a strong reducing agent (9% sodium thiosulphate), to break the disulphide bonds formed by the formalin fixation process. Partial tissue digestion was then performed, improving probe penetration further, using a 0.05% pepsin digestion at 37°C. This was followed by partial re-fixation in 10% formalin. Finally slides were dehydrated through an alcohol series.

For decalcified bone samples, a similar pre-treatment protocol was undertaken but using a manual method. Modifications for this method included the substitution of the tissue permeability and reducing agent steps with a customised antigen retrieval process (personal communication, M. Farquharson, Department of Pathology, Glasgow Royal Infirmary). Here, a pressure container containing one litre of antigen retrieval buffer was preheated (fifteen minutes at full power) in a microwave oven, the slides added to the solution and heated under pressurised conditions on full power. The pressure was released and the slides allowed to cool for twenty minutes before rinsing under cold water and further processing. Pepsin digestion for these samples was increased by 4 minutes compared to the soft tissue samples.

Once pre-treatment and tissue digestion were complete, all slides were examined for the adequacy of digestion (Edwards *et al.* 2001). Underdigested slides were digested further for a maximum of three minutes. Overdigested slides were discarded and duplicate slides digested for a reduced time (four minutes less per digestion for soft tissue samples and two minutes less per digestion for bone specimens).

Dual hybridization with an AR probe (SpectrumOrangeTM-labelled probe, locus Xql1-13, Vysis, UK, Ltd) and an X chromosome alpha satellite probe (Spectrum GreenTM-labelled CEP X, Vysis, UK, Ltd) was then performed in an identical way for bone and soft tissue specimens (Edwards *et al.* 2001). The AR probe used recognises a 3.4 MB complementary DNA sequence and the centromere probe a smaller 12kB DNA sequence.

Following hybridisation, adjacent serial haematoxylin and eosin (H&E) stained tissue sections were examined to delineate tumour areas. The DAPI-stained, FISH sections were examined on a Leica DMLB epifluorescence microscope fitted with a 100 W mercury arc lamp and Vysis triple and single band filters (Vysis 30-159080), that spanned the excitation and emission wavelengths of DAPI, SpectrumOrangeTM and Spectrum GreenTM. Twenty non-overlapping tumour nuclei were assessed per tumour section. Three different tumour sites were examined at x1000 magnification. The signals per nucleus for the AR gene and chromosome X were counted on a cell-by-cell basis. Nuclei with either no signal for AR or X or only one colour were not counted (Edwards *et al.* 2001). A second observer (Dr J. Edwards) scored the same tumour areas as the first observer (see **Appendix 3** for a example of a FISH scoring sheet).

AR gene copy number was calculated by totalling the number of orange signals (AR probe) counted in each specific area and dividing this figure by the number of nuclei assessed (see **Appendix 3**). The X chromosome copy number was calculated similarly. These results were combined to give a mean for each probe per tumour sample. The mean AR: X ratio was then calculated. AR gene amplification was defined as a mean AR gene copy number: mean X chromosome copy number ratio of greater than 1.5.

4.2.3 Statistics

The inter-observer error was calculated from the absolute difference between the means for each observer divided by the mean for both observers. If inter-observer error was > 20%, the specimen was reviewed again by the two observers.

Ninety-five percent confidence intervals (CIs) for the final score for each tumour sample were also calculated. Two-tailed Fisher's exact tests were used to compare chromosome X and AR copy number and AR gene amplification between the primary and HRPC tumour specimens.

The normal ranges for AR gene copy number and X chromosome number were derived from analysis of 14 benign prostatic hyperplasia (BPH) samples. A mean copy number +/- 3 standard deviations (SD) was used to produce 99% confidence intervals for both AR gene and X chromosome copy number (0.91-1.30 and 0.97-1.23 respectively). Abnormal AR gene copy number or chromosome X number was

defined as any figure outside these ranges. The normal range for the AR gene: X chromosome ratio in the 14 BPH samples was 0.93-1.07 (1.04 +/- 3SD).

4.3 Results

4.3.1 Patients

Altogether, samples from 29 different patients were collected prior to attempting FISH. These consisted of bone marrow trephine biopsies, post-mortem bone and soft tissue samples, TURP samples and a lymph node sample biopsy. Twenty-four samples could be analysed (18 trephine biopsies, 3 TURP samples, 2 post-mortem soft tissue samples and 1 supraclavicular lymph node biopsy, see **Table 4.3**).

FISH was successful in 18/24 (75%) cases (**Table 4.3**). Three bone marrow trephines contained insufficient tumour cells, as sixty tumour cells could not be identified confidently from within the sample (patients 6, 7, 21). In three autopsy samples (patients 10,12,16) repeated experiments failed.

Examples of FISH analysis for samples with normal AR gene and X chromosome copy number (**Figure 4.2**), twice the normal X chromosome copy number (**Figure 4.3**) and AR gene amplification (**Figure 4.4**) are shown.

The eighteen successful HPRC samples consisted of twelve bone marrow trephine samples, three local recurrence samples and three metastatic lymph node samples

(two autopsy samples and one Trucut® biopsy). The overall AR amplification rate for these eighteen HRPC cases was 9/18 (50%). The androgen receptor amplification ratios ranged from 1.66 to 5.09 (i.e. up to an average of 4.09 extra copies of AR gene per cell). Individual cell nuclei were scored as having up to 14 copies of the AR gene, but tight clusters of amplification made copy numbers difficult to determine precisely in some cases. For bone metastases, the AR gene amplification rate was 5/12 (38%).

For primary tumours, the AR amplification rate was 0/13 (**Table 4.3**). The highest AR: X ratio was 1.28. A significant difference between the AR gene amplification rates was observed between primary and HRPC cases, 9/18 compared to 0/13 ($p=0.0048$, Fisher's 2-tail exact, see **Table 4.3**).

Table 4.3

AR amplification ratio and aneusomy for chromosome X, 99% confidence intervals and inter-observer error rate for all HRPC samples and matching primary tumours (if available).

Sample No	HRPC: AR/X Amplification Ratio (95% CI)	I-O Variation	Primary Tumour: AR/X Amplification Ratio:(95% CI)	I- O Variation
1 (TUR)	4.67 (3.75-5.70)	15%	—	—
2 (TUR)	1.91 (1.65-2.17)	11%	—	—
3 (BMT)	1.1 (1.0-1.21)	4%	—	—
4 (BMT)	1.11 (1.07-1.15)	6%	—	—
5 (BMT)	5.09 (4.39-5.8)	5%	1.04 (1.02-1.05)	5%
6 (BMT)	—	—	1.02 (1.0-1.03)	6%
7 (BMT)	—	—	1.02 (1.0-1.07)	3%
8 (BMT)	1.16 (0.88-1.42)	13%	1.24 (0.97-1.51)	4%
9 (TUR)	1.02 (0.96-1.07)	7%	1.04 (1.02-1.07)	5%
10 (PM BM)	—	—	—	—
11 (BMT)	1.27 (1.17-1.39)	1%	1.14 (1.0-1.3)	1%
12 (PM BM)	—		1.07 (1.03-1.11)	9%
13 (BMT)	1.75 (1.22-2.35)	18%	—	—
14 (BMT)	2.07 (1.53-2.58)	5%	—	—
15 (PM LN)	3.10 (2.88-3.36)	14%	1.04 (0.87-1.21)	1%
16 (PM BM)	—		1.07 (0.95-1.21)	5%
17 (LN)	1.72 (1.63-1.81)	11%	—	—
18 (BMT)	2.53 (1.84-3.81)	10%	1.0	0%
19 (PM LN)	1.02 (1.0-1.05)	1%	1.12 (0.98-1.27)	8%
20 (BMT)	1.0	0%	1.21 (1.14-1.27)	4%
21 (BMT)	—	—	—	—
22 (BMT)	1.42 (0.69-2.06)	6%	—	—
23 (BMT)	0.99 (0.95-1.05)	0%	—	—
24 (BMT)	1.71(1.41-2.0)	16%	1.28 (1.17-1.39)	3%
Amplification Rate 9/18 (50%)		Amplification Rate 0/13 (0%)		

Table 4.3 (continued)

AR amplification ratio and aneusomy for chromosome X, 99% confidence intervals and inter-observer error rate for all HRPC samples and matching primary tumours (if available).

Sample No	HRPC:		HRPC:	
	Mean copy number AR (95% CI)	I-O Variation	Mean copy number X chromosome (95% CI)	I-O Variation
1 (TUR)	5.04 (4.04-6.04)	2%	1.08 (1.02-1.13)	14%
2 (TUR)	1.93 (1.64-2.21)	13%	1.01 (0.99-1.02)	2%
3 (BMT)	1.13 (1.0-1.27)	3%	1.03 (1.0-1.05)	2%
4 (BMT)	1.2 (1.08-1.32)	3%	1.08 (0.99-1.17)	9%
5 (BMT)	5.41 (4.64-6.19)	5%	1.07 (1.05-1.08)	0%
6 (BMT)	—	—	—	—
7 (BMT)	—	—	—	—
8 (BMT)	1.23 (0.85-1.62)	5%	1.06 (0.99-1.15)	6%
9 (TUR)	1.73 (1.25-2.2)	9%	1.7 (1.23-2.17)	16%
10 (PM BM)	—	—	—	—
11 (BMT)	1.31 (1.25-1.37)	4%	1.03 (0.98-1.07)	5%
12 (PM BM)	—	—	—	—
13 (BMT)	2.03 (1.52-2.55)	15%	1.16 (0.94-1.37)	1%
14 (BMT)	2.13 (1.56-2.72)	13%	1.03 (1.02-1.06)	11%
15 (PM LN)	3.88 (3.21-4.56)	3%	1.25 (1.12-1.38)	11%
16 (PM BM)	—	—	—	—
17 (LN)	1.87 (1.71-2.19)	5%	1.09 (1.01-1.26)	6%
18 (BMT)	3.65 (3.33-3.97)	13%	1.44 (0.67-2.21)	6%
19 (PM LN)	1.11 (1.04-1.17)	2%	1.08 (1.04-1.13)	0%
20 (BMT)	1.0	0%	1.0	0%
21 (BMT)	—	—	—	—
22 (BMT)	1.64 (0.56-2.73)	17%	1.15 (0.98-1.32)	12%
23 (BMT)	1.02 (0.98-1.05)	3%	1.03 (1.01-1.05)	3%
24 (BMT)	1.76 (1.42-2.09)	20%	1.03 (1.02-1.05)	3%
Total Abnormal AR copy number 12/18 (66%)		Total abnormal X chromosome number 3/18 (17%)		

Table 4.3 (continued) AR amplification ratio and aneusomy for chromosome X, 99% confidence intervals and inter-observer error rate for all HRPC samples and matching primary tumours (if available).

Sample No	Primary Tumour:		Primary Tumour:	
	Mean copy number AR (95% CI)	I-O Variation	Mean copy number X chromosome (95% CI)	I-O Variation
1 (TUR)	—	—	—	—
2 (TUR)	—	—	—	—
3 (BMT)	—	—	—	—
4 (BMT)	—	—	—	—
5 (BMT)	1.14 (1.08-1.21)	1%	1.1 (1.05-1.15)	3%
6 (BMT)	1.05 (1.02-1.08)	10%	1.03 (1.02-1.05)	3%
7 (BMT)	1.06 (1.04-1.07)	8%	1.03 (0.98-1.07)	5%
8 (BMT)	1.33 (0.88-1.77)	9%	1.07 (0.94-1.17)	5%
9 (TUR)	1.75 (1.04-2.31)	2%	1.68 (1.09-2.41)	1%
10 (PM BM)	—	—	—	—
11 (BMT)	1.29 (1.12-1.46)	6%	1.13 (1.07-1.18)	7%
12 (PM BM)	1.10 (1.05-1.15)	3%	1.03 (1.0-1.07)	6%
13 (BMT)	—	—	—	—
14 (BMT)	—	—	—	—
15 (PM LN)	2.16 (1.88-2.43)	7%	2.08 (1.97-2.20)	8%
16 (PM BM)	1.83 (1.09-2.58)	4%	1.71 (1.04-2.38)	3%
17 (LN)	—	—	—	—
18 (BMT)	1.0	0%	1.0	0%
19 (PM LN)	1.28 (1.0-1.56)	3%	1.14 (1.03-1.26)	10%
20 (BMT)	1.33 (1.29-1.61)	4%	1.1 (0.96-1.32)	0%
21 (BMT)	—	—	—	—
22 (BMT)	—	—	—	—
23 (BMT)	—	—	—	—
24 (BMT)	1.14 (1.29-1.61)	5%	1.45 (0.96-1.32)	1%
Total Abnormal AR copy number (5/13 (38%))		Total abnormal X chromosome number 4/13 (31%)		

Legend for Table 4.3

Normal range for chromosome X copy number (99% confidence intervals: 0.97-1.23). Normal range for AR copy number (99% confidence intervals: 0.91-1.30). AR Amplification defined as: Mean AR copy number / Mean X copy number Ratio >1.50.

AR; Androgen receptor, BMT: bone marrow trephine, HRPC; Hormone-refractory prostate cancer, I-O; Inter-observer, LN; Lymph node, No: Number, PM BM; Post mortem bone marrow, PM LN; Post mortem lymph node, TUR: Transurethral resection.

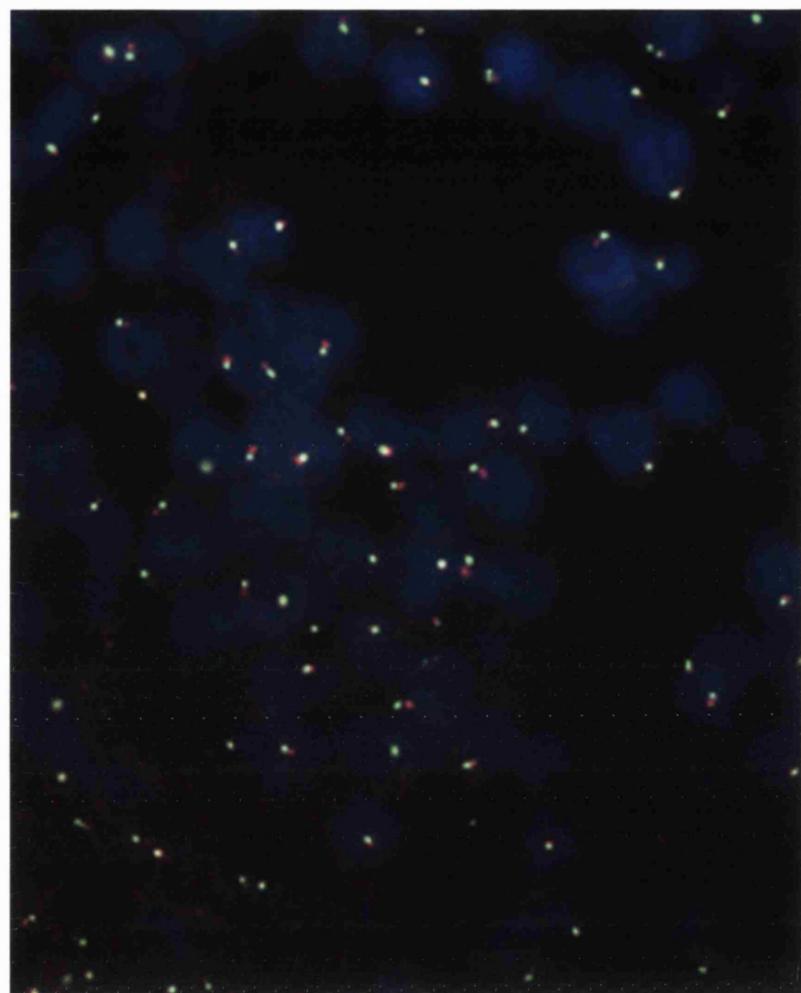


Figure 4.2

Section of primary prostate cancer showing nuclei stained with DAPI (4'-6-Diamidino-2-phenylindole-2HCl'). AR gene and X chromosome copy number were analysed by FISH using red (AR) and green (X chromosome) probes. Most nuclei contain one green and one red signal, indicating that each nucleus contains one X chromosome, on which there is one AR gene (x 100 magnification).

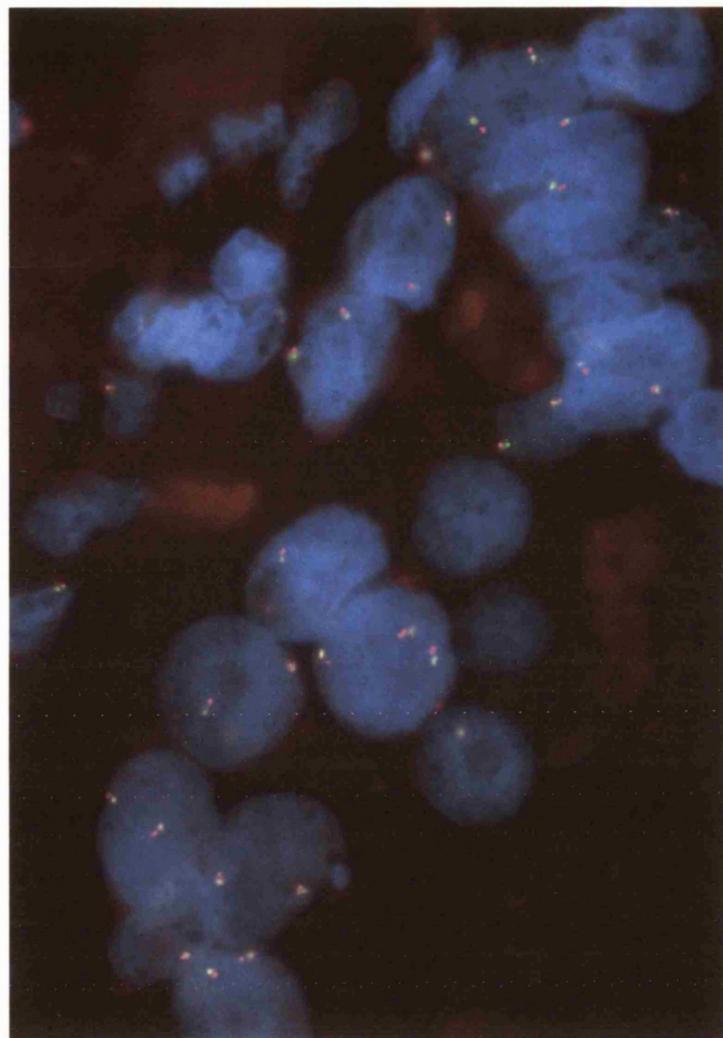


Figure 4.3

FISH section showing most cells in a primary tumour specimen containing two red (AR gene) and green (X chromosome) signals, indicating disomy (x 1000 magnification).

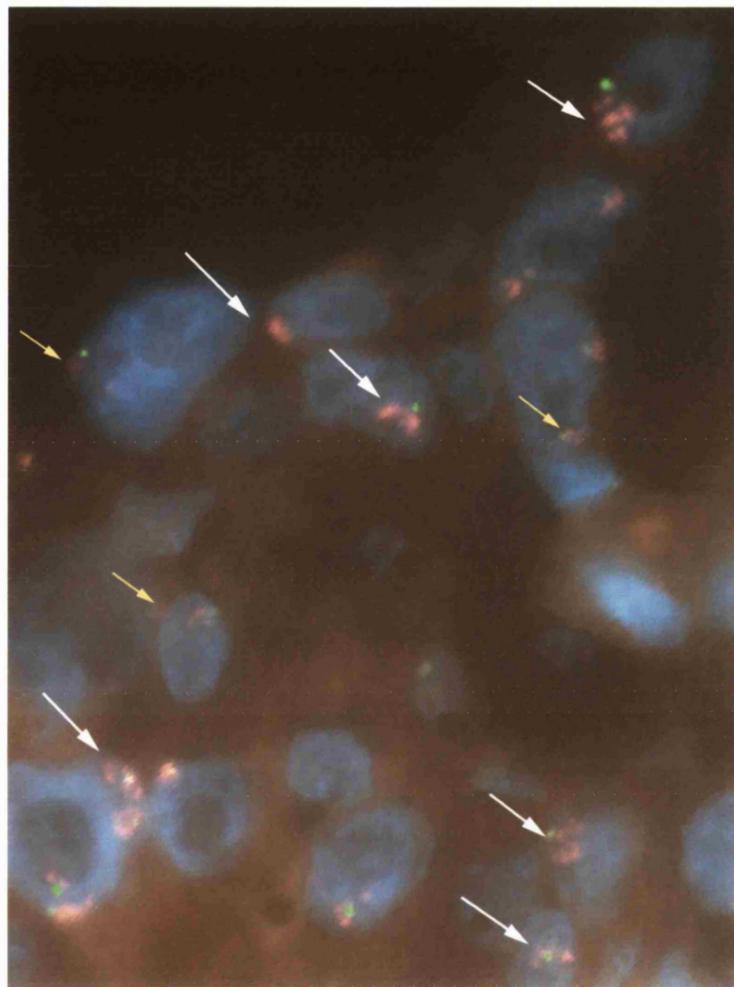


Figure 4.4

FISH section from an HRPC bone marrow sample, showing multiple red AR gene signals (white arrows) associated with a single X chromosome green signal. This indicates AR gene amplification. Note the glandular structure in the lower right corner of the picture and other areas of AR gene amplification not in the current plane of focus (yellow arrows). All at x 1000 magnification.

Elevation of the X chromosome copy number was observed in 4/13 primary tumour specimens (31%, mean X chromosome copy number 1.45, 1.68, 1.71 and 2.08) and 3/18 HRPC samples (17%, mean X chromosome copy number 1.25, 1.44 and 1.7). Three primary tumours (samples 9, 15 & 16) were disomic for the X chromosome (and AR gene) copy number. In one case (sample 9), both the primary tumour and the subsequent HRPC were disomic for the AR gene (rather than the normal single gene copy number). Another of these cancers (sample 15) developed AR amplification (mean AR: X ratio increasing from 1.04 to 3.10), associated with progression to HRPC. This was also associated with a reduction in chromosome X copy number from 2.08 per nucleus in the primary tumour to 1.25 in the HRPC specimen.

An elevated mean AR: X ratio without amplification (mean AR: X ratio between 1.07 and 1.5) was found in 5/18 (28%) cases of HRPC and 4/13 (31%) primary tumour samples. Only 4/18 (22%) HRPC samples had a normal mean AR: X ratio, compared with 9/13 (69%) primary tumours ($p=0.024$, Fisher's exact test).

Inter-observer error ranged between 0% - 19%, (**Table 4.3**). For primary tumours, the inter-observer errors were 10% or less in all cases. For HRPC samples, the corresponding figure was 49/67 (73%) observations with an inter-observer error of 10% or less and in 18/67 (27%) cases the inter-observer error ranged between 10-19%.

4.4 Discussion

The molecular mechanisms underlying the development of HRPC remain unclear. Androgen receptor gene amplification is found in a significant minority of cases of HRPC, and by allowing efficient utilization of low levels of circulating androgens may produce a growth advantage (Visakorpi *et al.* 1995; Koivisto *et al.* 1997). The AR signalling pathway remains functional in most cases of HRPC, both via AR signalling (Palmberg *et al.* 2000) and androgen-independent activation of AR (Sadar 1999).

These results show a higher frequency of AR gene amplification in HRPC than other studies, (50% compared with approximately 30%, range 0-36% (see **Table 1.2**) (Koivisto *et al.* 1995; Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Bubendorf *et al.* 1999; Kaltz-Wittmer *et al.* 2000; Miyoshi *et al.* 2000; Palmberg *et al.* 2000; Edwards *et al.* 2001; Haapala *et al.* 2001; Linja *et al.* 2001). It is apparent that AR FISH analysis is possible on most bone metastases, and levels of AR gene amplification (5/12, 38%) similar to other metastatic sites were found. This study was performed using routine histopathological sections, whereas in contrast, a small majority of studies have used isolated tumour nuclei for AR FISH (see **Table 1.2**) (Koivisto *et al.* 1995; Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Kaltz-Wittmer *et al.* 2000; Miyoshi *et al.* 2000; Palmberg *et al.* 2000). Tissue section material was required for a number of reasons. In most other AR gene amplification studies, the material examined has been from prostatic samples, where areas of high tumour load can usually be found. Such similar levels of tumour infiltration were not always seen in bone marrow sections we obtained. Additionally, tumour cells within bone marrow

are found against a background of rapidly dividing, non-cancerous normal cells of varying morphology. A method that ensured accurate identification of tumour cells within bone marrow samples was therefore required. By using tissue sections, slides could be related back to immediately adjacent haematoxylin and eosin stained sections, allowing accurate tumour cell identification under ultraviolet light.

Validation of these results against isolated tumour nuclei preparations, would have been useful but was not embarked upon because of the specificity problem. Two published comparisons using prostate tumour tissue for *in situ* hybridization, using both isolated nuclei and tissue sections from prostate cancer, suggest the methods provide similar data (Alers *et al.* 1995; Qian *et al.* 1996). Both have advantages and disadvantages. FISH analysis from isolated nuclei does not require special training in pathology, and signal counting is easier than in tissue sections (Qian *et al.* 1996). The method is also suited to large-scale studies by multiple investigators in a reasonable time frame. Tissue section analysis using FISH is slower and may suffer from nuclear truncation producing artificial monosomy.

In the small study by Alers *et al.* (eight tumours), section *in situ* hybridization was able to detect chromosomal alterations that were not seen in nuclear suspensions (Alers *et al.* 1995). Chromosome copy number changes, especially gains, were better seen in nuclear suspensions. The results show a higher frequency of AR gene amplification than usually reported in the literature, suggesting this was not a problem in our study. In addition to the two studies above that used tissue sections in comparison with isolated tumour nuclei, several other studies have used tissue

sections alone for *in situ* hybridization. Bubendorf *et al.* used tissue section microarrays in a large study that included AR gene amplification data from prostate tumour specimens and non-bony metastases (Bubendorf *et al.* 1999). Qian *et al.* and Alers *et al.* have both published studies using tissue section analysis for *in situ* hybridization (Alers *et al.* 1995; Qian *et al.* 1995). Our findings therefore show that AR gene amplification can also be reliably demonstrated in routine bone biopsy tissue sections, extending the potential of this tool in the management of HRPC.

There is no standard criterion for AR gene amplification (Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Bubendorf *et al.* 1999; Kaltz-Wittmer *et al.* 2000; Miyoshi *et al.* 2000; Palmberg *et al.* 2000; Edwards *et al.* 2001). In the present study a stringent definition of a ratio in excess of 1.5 (Edwards *et al.* 2001) together with a rigorous quality-controlled quantitation method (Watters *et al.* 1999) was used. Despite the strict definition for AR amplification, two cases were borderline (samples 13 and 24), in that the 95% confidence intervals straddle the definition of AR gene amplification (Mean AR: X ratio of >1.5). Such variability may simply represent inter-observer error. For the HRPC samples, 36/54 (66%) observations had an inter-observer error of 10% or less and in 18/54 (33%) cases the inter-observer error was between 10-19%.

Androgen receptor gene copy number can vary within individual tumours (Visakorpi *et al.* 1995). Heterogeneity of amplification was seen in one sample (mean AR: X ratios of 1.65, 1.95 and 2.53 for the 3 areas respectively). For sample twenty-four, two out of the three areas were scored as being amplified by both observers (mean AR: X 2.10 and 1.56). The remaining tumour area was scored as amplified by one

observer but not the other (with the average of the two areas being just >1.5). In a third sample (sample 22), the mean AR: X ratio did not reach 1.5 but the 95% CIs do include this figure. For this specimen, one out of the three areas examined showed AR amplification (mean AR: X ratio of 2.75) and aneusomy for chromosome X number (1.33) while the other two areas were normal for AR gene and X chromosome copy number. It remains unclear from this and other studies what level of increase in AR gene copy number may be associated with a biological advantage for tumours treated with androgen deprivation. However, if a subpopulation of cells within a HRPC develops AR gene amplification, this may be sufficient to provide a biological growth advantage and affect clinical outcome, despite not being recognised by our classification as being amplified.

The finding that AR gene amplification is rare in untreated primary tumours is in agreement with previously published reports (Koivisto *et al.* 1995; Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Bubendorf *et al.* 1999; Kaltz-Wittmer *et al.* 2000; Miyoshi *et al.* 2000; Edwards *et al.* 2001; Linja *et al.* 2001). In one primary tumour sample (sample 8), the upper limit of the 95% CI is above 1.5. The inter-observer error is small, and in two of the three areas examined there was no evidence of amplification. In the third area, possible low-level amplification was seen with a mean AR: X ratio of 1.52. The overall consistency of the findings that AR gene amplification is rare in untreated prostate cancer remains one of the strongest pieces of evidence that links AR gene amplification with the progression to HRPC. Only one study has suggested AR gene amplification in untreated primary tumours (8/17 cases, 47%, Ware *et al.* 2000).

Elevated copy number for chromosome X in HRPC has been previously reported in the range of 19-80% (Koivisto *et al.* 1995; Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Miyoshi *et al.* 2000; Edwards *et al.* 2001). Our results for HRPC samples are close to the lower end of this range (17%). An increase in X chromosome copy number without amplification has been postulated as a mechanism for the development of a proliferative advantage in tumours during anti-androgen therapy, mediated by increased expression of AR receptor levels (Koivisto *et al.* 1995; Koivisto *et al.* 1997). A more recent study has confirmed that one additional copy of the AR gene was able to increase AR protein expression in a human HRPC xenograft model (Linja *et al.* 2001).

The lowest success rate in FISH analysis was from autopsy material, in agreement with Bubendorf *et al.* (Bubendorf *et al.* 1999). For bone marrow post mortem specimens, there was a complete failure of all FISH analyses. This may be the result of acid hydrolysis of DNA in the decalcification process and similar problems can also occur in bone marrow trephine biopsies decalcified in hydrochloric acid. (Afers *et al.* 1999). In contrast, decalcification in five percent formic acid overnight, used in this study allowed successful FISH analysis. The standard departmental decalcification protocol (10% formic acid for 7-10 days) for post mortem bone samples resulted in failure despite successful FISH in soft tissue autopsy material from the same patient.

Studies of the genetic and molecular changes in androgen-related gene expression in metastases from HRPC may be important for the management of these patients (Bubendorf *et al.* 1999). Tumours with AR gene amplification may represent a

subset of HRPC that might be targeted by other potential treatments, for example antisense therapy (Eder *et al.* 2000; Devi 2002; Eder *et al.* 2002), geldanamycin (Solit *et al.* 2002; Vanaja *et al.* 2002) or geldanamycin-testosterone hybrids (Kuduk *et al.* 2000). A recent study has suggested that AR gene amplification in local tumour recurrences at progression following androgen deprivation monotherapy is associated with a better response to second line combined androgen blockade (Palmberg *et al.* 2000). Growth of tumour cells exhibiting AR gene amplification may be dependent on residual androgens. This study also suggests there may be a clinical benefit for patients in identifying AR gene amplification upon progression to HRPC (Palmberg *et al.* 2000). The authors noted that sampling in this context should be done at metastatic sites, but obtaining such tissue was a challenge in developing predictive tests based on AR gene amplification. Bone marrow sampling was suggested as a method of achieving this. We have shown here, and in the previous chapter, that bone marrow samples can be obtained routinely from HRPC patients and analysed by FISH for AR gene amplification. Studies relating AR gene amplification rates in bone metastases to clinical outcome following the addition of combined androgen blockade treatment are needed.

Chapter 5:

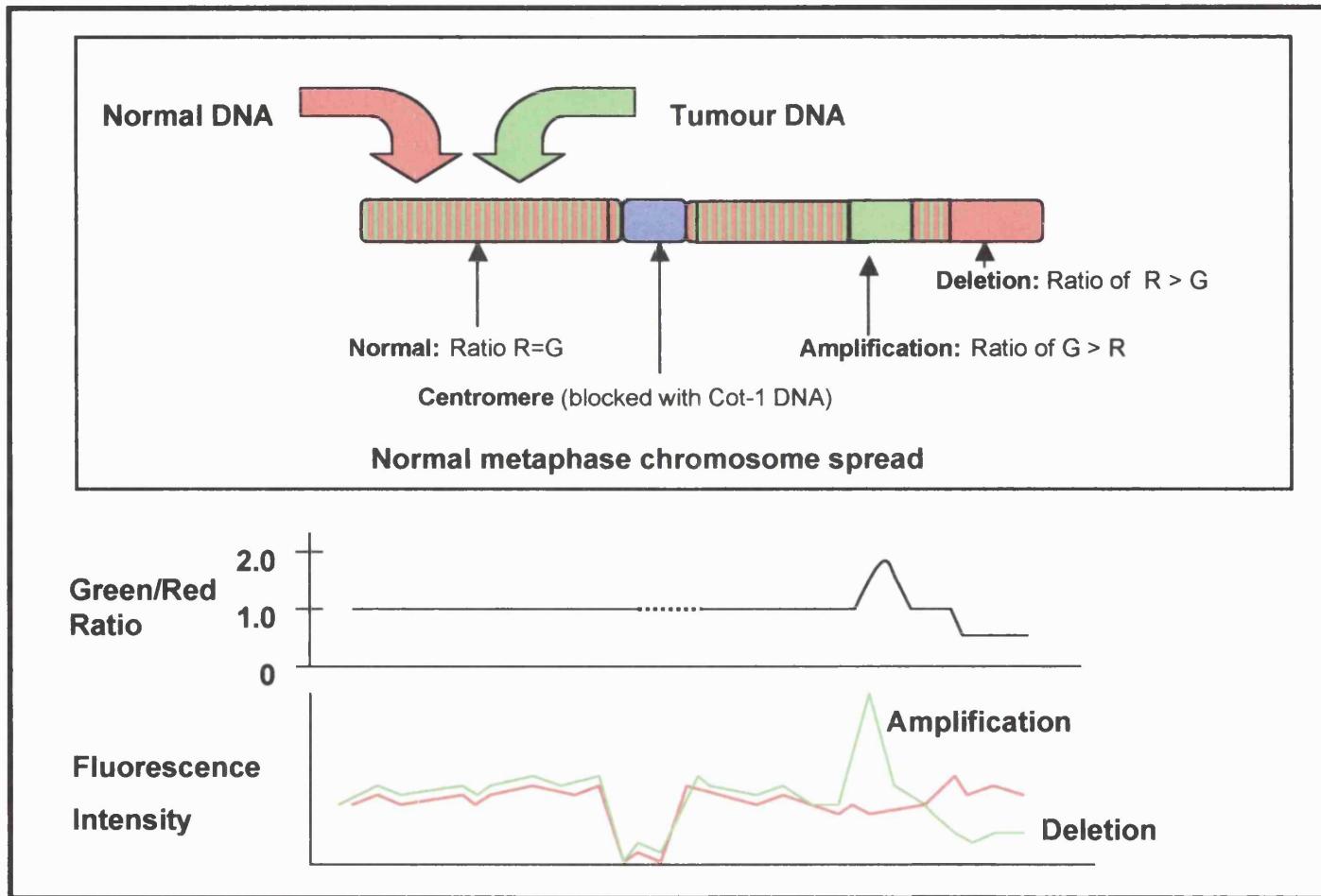
**Comparative Genomic Hybridization studies in hormone-
refractory prostate cancer samples**

5.1 Introduction

Comparative genomic hybridization was first described by Kallioniemi *et al.* in 1992 (Kallioniemi *et al.* 1992). Development of the technique was said to be motivated by an expectation that improved detection of gene amplifications, other DNA gains as well as DNA losses and gene deletions would be important in cancer research (Kallioniemi *et al.* 1994).

CGH introduced rapid, quantitative assessment of genome-wide losses and gains in DNA content. Based on two coloured FISH, the procedure used competing, simultaneous hybridizations of a normal reference rhodamine-red fluorescent DNA and a FITC-green fluorescent tumour DNA, against a normal human metaphase chromosome spread (seen in **Figure 5.1**). The relative amounts of tumour and reference DNA bound at any given chromosomal locus, depends upon the relative amounts of the sequences within the competing DNA samples. A ratio of red to green fluorescence can be produced from this hybridization using a computerised QUantitative Image Processing System (QUIPS, developed at the University of California, San Francisco and the Medical Research Council Human Genetics Unit, Edinburgh, Piper *et al.* 1995). Amplifications of genetic material within a tumour sample are represented by an increased green to red signal ratio. Loss of genetic material produces a decreased green: red ratio. Areas containing similar quantities of normal and tumour DNA return a green: red ratio of near equal proportion.

Figure 5.1 Schematic representation of CGH



See over page for Legend

Figure 5.1 Schematic representation of CGH.

Normal DNA (labelled with red fluorophore) and tumour DNA (green fluorophore) are simultaneously, competitively hybridized to a normal human metaphase preparation. Losses of DNA results in a predominantly red hybridization signal and amplifications in regions of DNA gain are seen as increased green signal.

The intensity of the red and green fluorescence along the central axis of each chromosome is measured by the digital imaging analysis program. The fluorescence ratio (red: green) gives a quantitative estimate of copy number variations within the tumour DNA.

The use of Cot-1 DNA included at the time of hybridization partially blocks the labelling of fluorophore-labelled DNAs at the centromeric repeat regions.

(Adapted from Kallioniemi *et al.* 1993).

Apart from the genome-wide application of CGH, other advantages of the technique include; no specific prior knowledge of any genetic locus or loci is required, and that a specific probe need not be developed to start searching for genetic abnormalities. The technique also accurately maps abnormalities to recognizable chromosomal regions, providing a starting point for locus specific identification techniques such as FISH. Semi-quantitative estimates of amplification levels can be provided by CGH where the height of the ratio profile peak estimates the level of amplification (low- or high-) and the width of the peak gives information on the total size of the amplicon unit.

The sensitivity of CGH for detecting genomic amplifications is approximately 5-10 fold gene amplifications or 10-20 megabases of DNA for lower level copy number gains. Sensitivity depends upon the amplicon length. The larger the amplified segment, the lower the level of amplification that may be detected (Kallioniemi *et al.* 1992). A high level amplification may be as little as 1-2 megabases of DNA in length (e.g. a 10-fold amplification of a 200 kb region) and be detectable by CGH (Kallioniemi *et al.* 1994). Sensitivity for detection of deletions and losses is poorer and is similar to that of low-level amplifications (i.e. 10-20 megabases).

A number of recognised limitations in CGH have been identified. CGH only detects genetic aberrations that cause loss or gain of DNA sequences. Balanced translocations, inversions, point mutations and intragenic rearrangements cannot be detected by this method.

CGH can only detect DNA copy number changes relative to the average copy number within an entire tumour sample. Therefore, triploid or tetraploid chromosomal content cannot be distinguished from a diploid number (this would require concurrent FISH to independently identify copy number at several different loci). Genetic heterogeneity is also known to occur within tumours (Visakorpi *et al.* 1995) and may also be under-represented by CGH. Homogeneous (clonal) aberrations are more readily detected, but genetic changes in bi-clonal or multi-clonal tumours may balance one another.

Due to the way that chromosomal DNA is conveyed within the denatured metaphase chromosomes (and the way fluorescence profiles are generated by integrating fluorescence across the chromosome), colour ratios from amplified regions spread over larger regions than the true amplicon. Semi-quantitative information rather than exact numerical accuracy about the level of amplification is only available.

The hybridization of labelled-DNAs to problematic areas of the genome requires blocking with Cot-1 DNA. Centromeric and heterochromic regions contain differing polymorphisms between individuals (i.e. normal-normal CGH hybridizations in these regions show substantial variation in green: red fluorescence ratios). These areas are not completely blocked by the Cot-1 DNA and centromeric and heterochromic areas are excluded from the analysis. Green and red fluorescence also gradually decrease at chromosomal telomeres. Colour ratios at these regions also need to be interpreted with caution. Certain other areas of the genome may also be prone to locus specific ratio fluctuations (below average green: red fluorescence ratios). These may be

interpreted as false positive deletions. Chromosomal regions at 1p32-pter, 16p, 19 and 22 have exhibited this problem and need to be excluded from the analysis.

Normal cell contained within a sample may dilute the genetic changes within a tumour. Kallioniemi *et al.* have however previously shown that 30 to 50% dilution by a normal cell population can still provide useful information on copy number changes (Kallioniemi *et al.* 1994).

CGH cannot provide direct information on the identity of affected genes within a region of deletion or amplification, but acts as a starting point for the isolation of the precise nature of the change in the affected areas.

A number of standard CGH studies of prostate cancer have now been performed on primary tumours, recurrent tumours, hormone-refractory tumours and untreated metastases (Wolter, Gottfried *et al.* 2002, Wolter, Trijic *et al.* 2002, Kasahara *et al.* 2002, Verdorfer *et al.* 2001, Chu *et al.* 2001, Happala, Rokman *et al.* 2001, Mattfeldt *et al.* 2001, Alers *et al.* 2001, Sattler *et al.* 2001, Rokman *et al.* 2000, Sattler *et al.* 1999, Nupponen *et al.* 1998, Alers *et al.* 1997, Cher *et al.* 1996, Joos *et al.* 1995, Visakorpi *et al.* 1995).

Following the first report of CGH, modifications and variations in the technique have been published. One of the most important of these has been the ability to perform CGH on formalin-fixed, paraffin embedded archival material (Speicher *et al.* 1993, Isola *et al.* 1994). Formalin-fixed, paraffin-embedded archival material allows clinically orientated and prognostic studies to be undertaken (Isola *et al.* 1994). This

development also widened substantially the number of samples that could be studied. Tumour pathogenesis can be studied by mapping genetic progression from premalignant to malignant lesions or by comparison of the abnormalities in primary tumours and their metastases. It may prove possible to develop prognostic information from formalin-fixed paraffin-embedded CGH studies.

Conventional CGH requires approximately 1 µg of sample DNA, corresponding to approximately 100,000 diploid cells (Kallioniemi *et al.* 1994). A CGH method that used significantly smaller DNA samples was first published by Speicher *et al.* in 1993 (Speicher *et al.* 1993). Universal amplification of DNA by the degenerative oligonucleotide primer-polymerase chain reaction (DOP-PCR) method was described, increasing sample DNA levels prior to fluorescence labelling. DOP-PCR has been shown to produce sufficient amplified DNA for CGH from as little as 50 pg of template DNA (Huang *et al.* 2000). A single cell CGH method has also been described by Klein *et al.* (Klein *et al.* 1999, Klein *et al.* 2002).

Other DOP-PCR CGH studies have used paraffin embedded prostate cancer samples (Chu *et al.* 2003, Steiner *et al.* 2002, Beheshti *et al.* 2002, Zitzelberger *et al.* 2001, Verhagen *et al.* 2000, Kim *et al.* 2000, Kim *et al.* 1999, Zitzelberger *et al.* 1998). No studies have yet to report DOP-PCR CGH on bone metastases in prostate cancer (including those with hormone-refractory disease). The technique may provide a useful way to screening bone metastases from HRPC patients for genetic alterations.

5.2 Methods

The work was performed with the guidance of Dr Chris Jones at the Department of Pathology, University College London. The standard operating procedure is listed fully in Appendix 2. Briefly, the methodology consists of preparing paraffin-embedded, formalin-fixed, 5 µm tissue sections on plain microscope slides for laser capture microdissection. Laser capture microscopy is then performed, isolating and collecting near-pure prostate cancer and normal cell populations from the bone marrow sections.

DNA was extracted with a Proteinase-K tissue digestion. Non-specific DNA amplification was performed using a first DOP-PCR for all samples. Amplified DNA products were checked for size using an ethidium bromide/agarose gel (DNA containing fragments of > 1000 bp are required for successful CGH). If DNA quality was sufficient, the samples were taken forward to a second fluorescence-labelling DOP-PCR, producing the hybridization probes. Further LCM and DNA extraction was required if DNA product length was poor.

The genome-wide DNA probes were hybridized to commercially-produced, normal human metaphase chromosomes (Vysis, Europe), followed by a 72 hours incubation at 37°C in lightproof conditions. Slides were washed to remove unbound probe, counterstained with DAPI to produce a faint R-band pattern, and the specimens sealed underneath a coverslip.

Slides were visualised on a fluorescence microscope (Zeiss Axioskop with a triple band bypass filter). If the hybridizations are of adequate quality, they are captured as a series of multi-coloured (red, green and blue), digital images for investigation with QUIPS® analysis software (Vysis, Europe). Identification and manual pairing of chromosomes images is performed prior to performing the fluorescence ratio analysis. The analysis is based upon the contrast-stretched, digital images and green: red fluorescence intensity ratio profiles. Copy number differences in the tumour and normal DNA are produced for each chromosome pair. For visual assessment, the three single-coloured images are overlaid and displayed in pseudo-colour (approximating the original colours of the fluorophores and DAPI counterstain). Profiles from several metaphase spreads are combined, improve the signal to noise ratio and the accuracy of the technique. Profiles of the mean ratio ± 1 standard deviation (S.D.) are then calculated. In a successful hybridization, this normal variation (± 1 S.D.) should not exceed ratios between 0.85 and 1.15.

5.3 Results

Laser Capture Microscopy

Initial LCM slides were made from 23 of the 29 patients samples. All 23 samples were HRPC specimens, and consisted of 14 bone marrow samples, 5 post mortem samples, 3 TUR samples and 1 lymph node sample.

LCM was performed on all samples. **Figure 5.2** shows an example of tumour cells extracted from a bone marrow trephine specimen from patient 14. Separate samples of normal tissue and tumour were microdissected and individually captured. A total of 46 tissue caps were made.

DNA Extraction and 1st DOP-PCRs

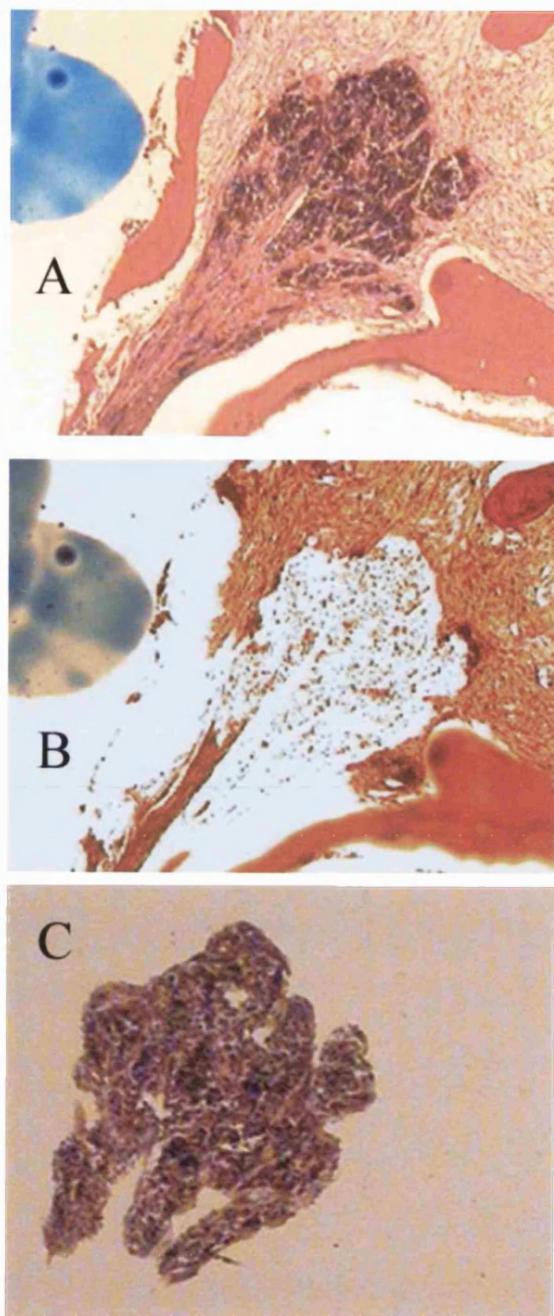
Tissue DNA extraction was performed on all samples. In total, after DNA extraction and the first DOP-PCR, suitable fragment lengths for the second labelling DOP-PCR were obtained in 20 / 23 tumour samples (87%) and 15 / 23 cases from normal tissues (65%).

Of the 14 bone marrow trephine samples, paired tumour and normal DNA were extracted and amplified in 10 / 14 cases. In a further 3 cases, tumour but no normal DNA samples could be obtained, giving a total of 13 /14 successful tumour DNA extractions (with a single trephine sample producing no tumour or normal DNA). From the 5 post-mortem samples, paired samples were obtained in 1 case and tumour

DNA alone in a further 2 cases. No suitable DNA was retrieved in the remaining 2 cases. The 3 TUR samples and the lymph node sample produced satisfactory DNA sample pairs.

Figure 5.3 shows an agarose gel containing some of the extracted and amplified DNA samples checked for suitability of product length.

Figure 5.2



Legend for Figure 5.2.

Images from the laser capture microscope of decalcified, formalin-fixed, paraffin-embedded tumour cells from a bone marrow trephine specimen.

A. Pre-capture image showing a tongue of tumour tissue in between 2 areas of pink trabecular bone. The blue ink, adjacent to the tumour, indicates tumour areas as marked by the histopathologist (Dr Ahmet Dogan) prior to performing LCM (x 400 magnification).

B. The same sample showing remaining tissue after laser capture. Note the left area of trabecular bone has also detached and required removal by the CapSure® adhesive pad before DNA extraction (x 40 magnification).

C. High power view of the CapSure® cap containing the laser-captured tumour area ready for DNA extraction (x 600 magnification).

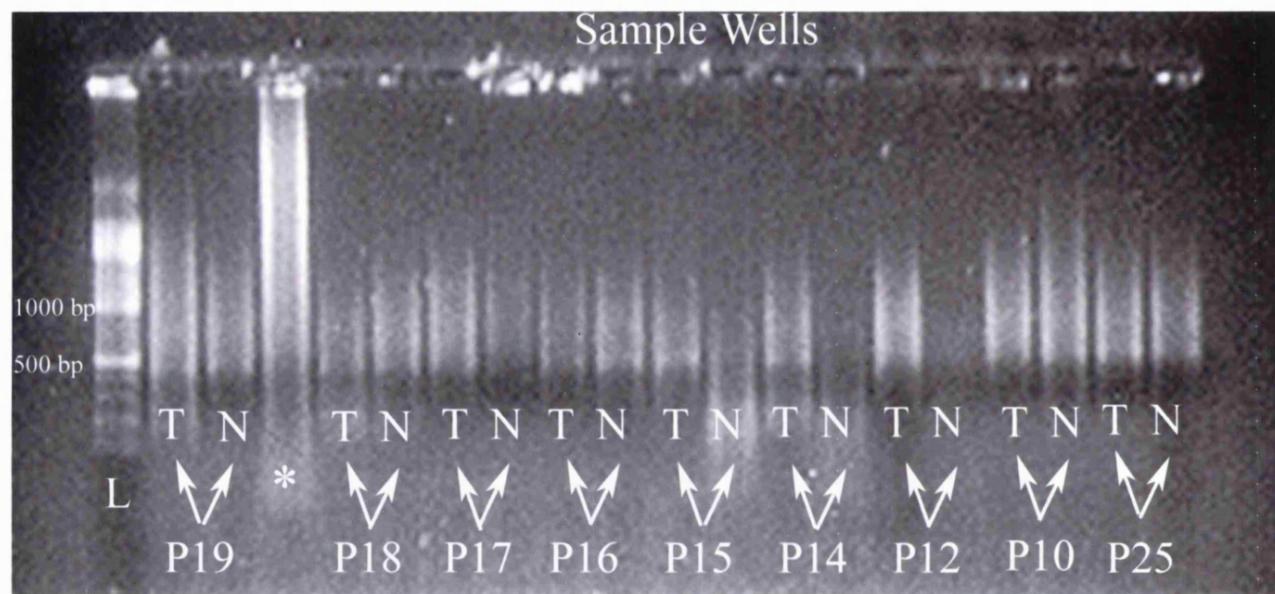


Figure 5.3

Legend for Figure 5.3

Agarose electrophoresis gel showing DNA smears derived from formalin-fixed, paraffin embedded tumour and normal tissue (with additional decalcification processing step for bone marrow trephines).

Amplified DOP-PCR products were run from the sample wells concurrently with a ladder of DNA of known standard sizes (500 and 1,000 base pair markers are labelled on the ladder, L, at the top of the figure). T: tumour DNA smears, N: normal DNA smears. P followed by a 2-figure number identifies which patient samples are shown. P19, 18, 17, 16, 10 and 25 all show satisfactory DNA smears able to be taken forward to a second labelling DOP-PCR. P14 and P12 show satisfactory tumour smears but poor normal DNA smears.

The * shows a normal DNA smear from patient 20 of very poor quality (and no tumour sample could be obtained from the trephine section at LCM).

Second DOP-PCR and Metaphase Hybridizations

In total 10 successive hybridization experiments were performed. All paired samples of tumour and normal DNA were taken through the second DOP-PCR fluorescence labelling and metaphase hybridization over the 5 initial experiments. None of these samples produced sufficiently high quality hybridizations for image analysis. In the first experiment, no hybridization signal was seen, a recognised failing (personal communication, Dr Chris Jones). These samples underwent repeat labelling DOP-PCR and hybridization producing a visible signal. The third, fourth and fifth CGH experiments also showed fluorescent signal from the probes. In many of the sections, good quality tumour hybridizations were achieved (see **Figure 5.4**) but these were associated with poor normal DNA hybridizations (see **Figure 5.5A**). A number of other samples in these experiments also showed high patchy background fluorescence (**Figure 5.5B**). Image analysis from any of these samples was not possible. In other cases, tumour DNA hybridization signal was visibly inconsistent across the genome (**Figure 5.5C**), also making them unsuitable for analysis. Others hybridizations were very dull and washed out in appearance (**Figure 5.5D**). Apart from the repeated failure to achieve adequate normal DNA hybridizations, problems with background fluorescence, inconsistent tumour labelling across the genome and dull hybridizations were idiosyncratic and did not show any discernable repeating pattern.

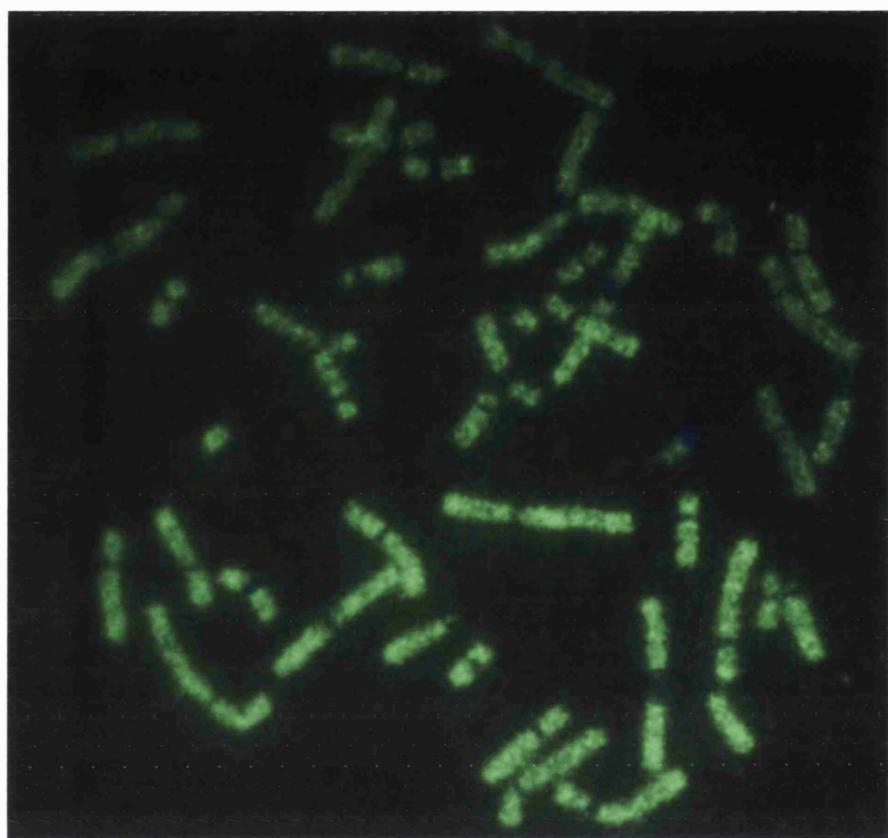


Figure 5.4. Successful tumour DNA hybridization (green fluorescence) to a metaphase spread.

In CGH experiments six and seven, standard hybridizations using some of the previously tested samples were repeated. No modification of the methodology was undertaken, but by now there was good familiarity and fluency with the technique. Results from these experiments however continued to show the combination of poor normal DNA hybridization, unpredictable high background fluorescence and inconsistent tumour signal across the genome (**Figure 5.5A-C**).

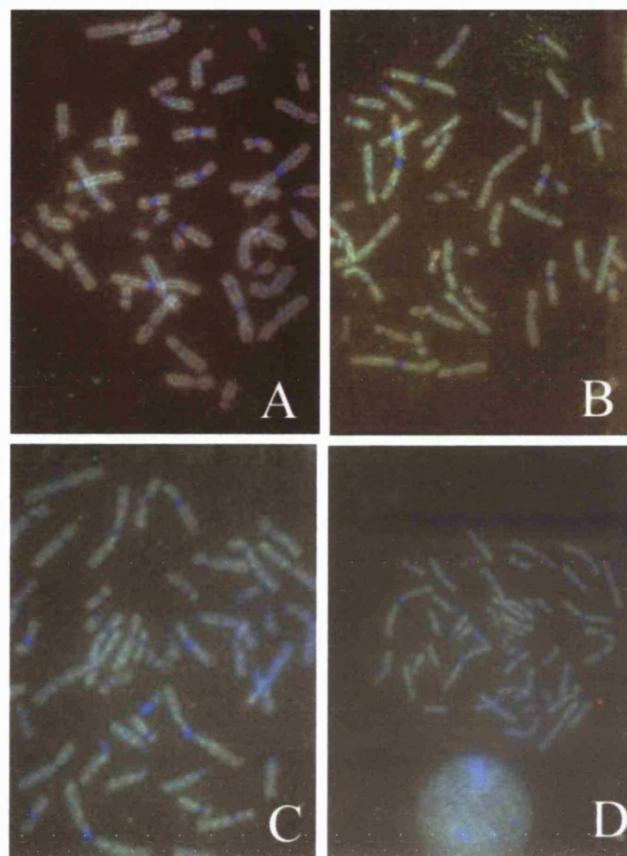


Figure 5.5:

Examples of CGH hybridization of insufficient quality to take through to image analysis:

- A.** Poor normal (red) DNA hybridization.
- B.** Speckled Fluorescent background staining.
- C.** Inconsistent tumour DNA (green) hybridization across the chromosomes.
- D.** Dull image.

CGH experiment 8 performed a hybridization using a tumour sample without a matching normal DNA, against a normal DNA sample produced from frozen tissue, labelled by the nick translation (rather than DOP-PCR). This was to test the hypothesis that changing the labelling method for the normal DNA might improve the quality of the overall hybridizations. The experiment failed to show sufficient normal DNA hybridization for analysis.

In the final two experiments, commercially available (SpectrumRed®, Vysis, UK), nick-translated, normal DNA for hybridizations was also performed. In the first of these, a non-comparative hybridization using the SpectrumRed® probe alone was performed. This produced the best quality normal hybridization seen (see **Figure 5. 6**) but there was still evidence of patchy hybridization across the genome on a few occasions. A further CGH experiment was performed hybridizing a tumour sample (that had previously produced good quality signal), with the SpectrumRed® normal DNA sample. This experiment also failed to produce any hybridizations of sufficient quality for analysis. At this point the experiments were abandoned in favour of work reported in the next chapter.

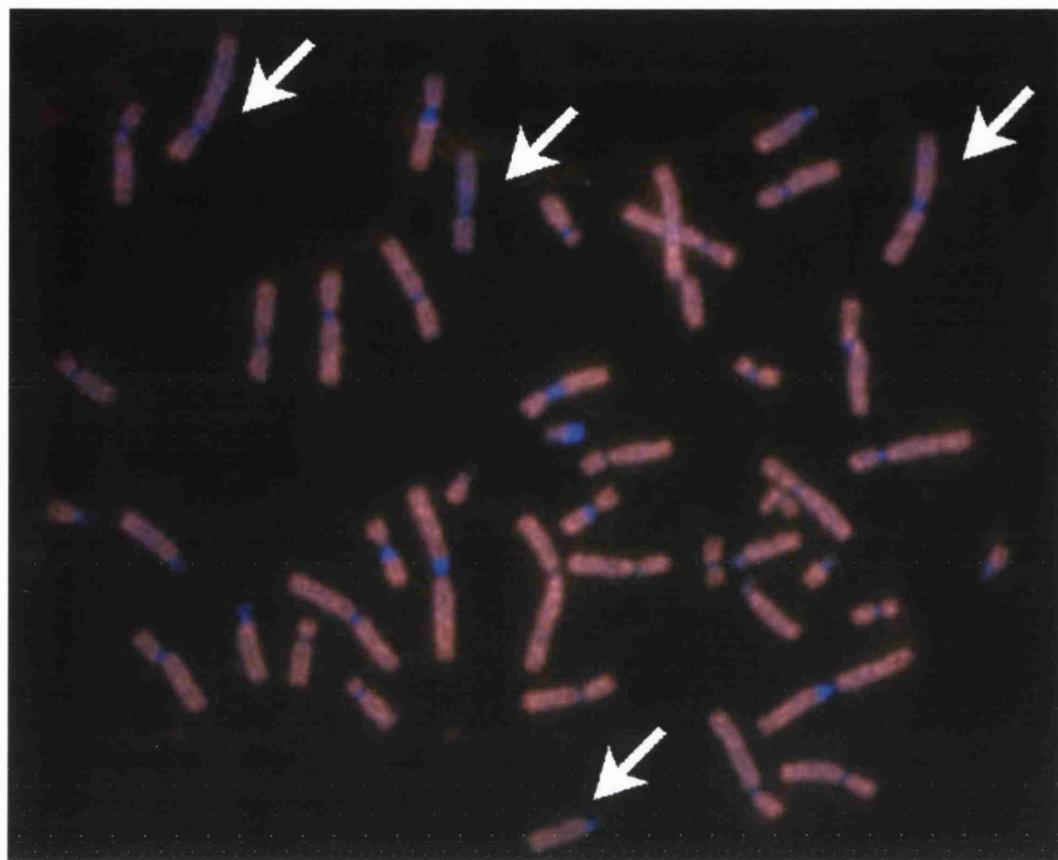


Figure 5.6

Single hybridization experiment using commercially available SpectrumRed® normal DNA to a metaphase spread. Note although hybridization is generally good, there are some areas of lower signal intensity (white arrows).

5.4 Discussion

It is recognised that establishment and routine application of CGH is demanding and requires fine-tuning of methods for each step of the protocol (Kallioniemi *et al.* 1994). The quality of the CGH analysis is dependant on the characteristics of the metaphase spread and high-quality hybridizations cannot always be achieved (Kallioniemi *et al.* 1994). DOP-PCR CGH is also reported to be complicated and less reliable than traditional CGH (Nupponen & Visakorpi 2000).

DOP-PCR CGH was required for this study because of the nature of the specimens obtained. The samples contained a mixture of highly active normal bone marrow precursor cells as well as tumour deposits. In some samples only small amounts of tumour occurred within the normal background cell population (**Figure 5.2**).

Initial studies using DOP-PCR suggested reference DNA need not come from the same person as the tumour (Kallioniemi *et al.* 1993). In contrast, Huang *et al.* and James and Varley have suggested that reference DNA must be amplified by DOP-PCR for optimal CGH (Huang *et al.* 2000, James & Varley 1996). I have been able to extract and amplify normal DNA in a proportion (65%) of cases, but could not translate this into hybridizations of sufficient quality to analyse with the QUIPS software. The addition of commercially labelled normal DNA gave better hybridizations (**Figure 5.6**) but still did not allow successful CGH in the final CGH experiment performed.

Differences in fixation protocols may have contributed to difficulties in the CGH. Tissue samples were obtained from 6 different laboratories for use in the CGH. Extensive cross-linking of nuclear proteins, formation of tight complexes between proteins and DNA and fragmentation of DNA are all known to occur because of formalin fixation methods. CGH on freshly frozen material is said to be more reliable (Kallioniemi *et al.* 1993). However dual-colour hybridization were able to be performed on many of the samples using locus specific hybridization (FISH), as shown in the previous chapter, suggesting it is less likely that fixation problems were a major cause of the hybridization failures.

DOP-PCR amplified fragments are known to be smaller from microdissected, formalin-fixed, paraffin-embedded samples (100 base pairs to 2 kb, Huang *et al.* 2000). It is also known that the greater the degradation of the starting DNA, the smaller the DNA fragments produced. Severely degraded DNA cannot be used for CGH and this is the likely reason why some post mortem bone samples could not be used to produce adequate DNA fragment lengths.

There are two studies in the literature using samples of formalin-fixed, paraffin-embedded archival bone metastases (following repeated Medline searches on prostate cancer and all bone marrow CGH reports). Alers *et al.* described specific changes in chromosome eight, seen in three of six archival bone metastases analysed by *in situ* hybridization (Alers *et al.* 1997). These samples were included in a larger series of prostate cancer cases. CGH was subsequently performed on four of the bone metastases, identifying the amplifications as consisting of the whole arm of

chromosome 8q in 2 cases, and most of the 8q arm in the third case (8q13 to 8qter). These finding were not seen in any of the other sites of metastases analysed. Alers *et al.* used a manual microdissection method; suggesting relatively large amounts of material were available for study (20-30 5 μ m paraffin sections are required to extract 1-2 μ g of genomic DNA for conventional CGH, Isola *et al.* 1994). An initial DOP-PCR process was therefore not required to amplify the genetic material. DNA extraction was followed by fluorescence labelling with nick translation, rather than the second fluorescence-labelled DOP-PCR method required to be used here. Probe sizes obtained were between 300 bp and 1.5 kb for both tumour and normal DNA, similar to the results I obtained. Hybridization of probes to normal human metaphases was performed in the same way as described by Kallioniemi (Kallioniemi *et al.* 1992) and used in the experiments here.

It was not stated in the study by Alers *et al.* how the bone samples were decalcified, but the effects of decalcification on their CGH was explored in a subsequent paper (Alers *et al.* 1999, see also Chapter 3). This suggests that the bone samples had been decalcified using EDTA solution, as the subsequent paper showed that none of eight acid-decalcified specimens resulted in successful CGH while in comparison, three successful CGH experiments were performed on EDTA-decalcified samples (where DNA ranging up in size up to 10 kb could be extracted from both normal and tumour material).

Five percent formic acid decalcification (as used in the samples here) resulted in suitable tumour DNA smears being obtained for 13 out of the 14 trephine samples (see **Figure 5.3**), and it was possible to hybridize tumour DNA successfully to metaphase spreads in some cases (**Figure 5.4**). Samples that did not undergo decalcification were no more or less consistent than bone marrow derived DNA. The additional decalcification step alone therefore does not explain why it was not possible to produce good quality hybridizations for the CGH experiments. The ability to perform hybridizations using dual-colour fluorescence (as outlined in the previous chapter) also suggests a major effect of decalcification with five percent formic acid was not the principle cause of the CGH failures. I am unable to exclude some subtler affect of acid decalcification on the hybridization quality, as none of the samples obtained were decalcified using EDTA.

The second study in the literature that used formalin-fixed, paraffin-embedded bone marrow tissue reported on ten cases of acute myeloid leukaemia (Heller *et al.* 2000). In each case, the authors performed DOP-DCR on fifteen interphase nuclei from a fixed bone marrow aspirate suspension, microdissected from a cover slip. Decalcification of the specimens was not required. This method was not technically possible to use here as the aspirate samples were not shown to contain tumour cells and no attempt to obtain smears from the bone marrow trephine at the time of collection was performed. When collecting the samples (May 1999-June 2000), this paper had not been published.

The most likely cause of the failure of the hybridizations is operator inconsistency, given the range and different types of technical problems encountered. After a number of months work on the CGH, I decided against further attempts to improve the hybridization quality because of time constraints.

Chapter 6:

**Can immunohistochemistry predict androgen receptor gene
amplification demonstrated by FISH?**

6.1 Introduction

The previous chapter has demonstrated that FISH can be used to determine the presence of androgen receptor gene amplification in bone metastases from HRPC. Preliminary evidence suggests that this may be of clinical value (Palmberg *et al.* 2000). However, FISH is a specialised molecular technique, not generally available in most diagnostic pathology departments. This may limit its usefulness. In contrast, immunohistochemistry (IHC) is a widely available technique that is routinely used in diagnostic pathology (Miller 2000). There is evidence that AR gene amplification results in overexpression of the AR protein (Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Linja *et al.* 2001). Therefore, it may be possible to correlate levels of AR protein expression, as measured by IHC, with the presence or absence of AR gene amplification and widen the scope for diagnostic use. The most important limitation is likely to be the difficulty of quantifying immunohistochemical staining and this obstacle may preclude the accurate comparison of levels of expression in archival tissue specimens (Visakorpi *et al.* 1995).

Immunohistochemistry is used to assess over-expression of the c-erbB-2 (HER-2/neu) receptor resulting from *c-erbB-2* gene amplification in breast cancer. The United States Food and Drug Administration has licensed the IHC method (Dako HerceptTest®, Carpinteria CA, USA) as a recommended method of demonstrating HER-2 overexpression in archival, paraffin-section, breast cancer material. Over-expression can be demonstrated either by using FISH to detect the amplification of the gene or by IHC to detect the protein expression (Lebeau *et al.* 2001). It is known

that *cerbB-2* gene amplification consistently results in overexpression of mRNA and protein (Tsuda 2001).

A further parallel with breast cancer exists in that immunohistochemistry is currently the most widely used method for assessing steroid hormone (oestrogen, ER and progesterone, PR) receptor status. These receptors (particularly ER) have important prognostic and therapeutic implications in the treatment of breast cancer (Early Breast Cancer Trialists' Collaborative Group 1998; Spiers *et al.* 2000). Although AR status has not been shown to have the same strong prognostic and therapy implications in prostate cancer as ER and c-erbB-2 status in breast cancer (Reiner *et al.* 1990; Spiers *et al.* 2000), there is evidence to suggest AR status, as demonstrated by IHC, has prognostic significance (Pertschuk *et al.* 1994; Pertschuk *et al.* 1995; Takeda *et al.* 1996). Other results have however been contradictory (Sadi *et al.* 1991) and the goal of the early 1990s to determine whether the androgen receptor could be used to predict clinical response of metastatic prostate cancer to androgen deprivation therapy has not been realised in a similar way to that of ER/PR status in breast cancer.

Androgen receptor has been extensively studied in prostate cancer using IHC (Masai *et al.* 1990; Sadi *et al.* 1991; van der Kwast *et al.* 1991; Brolin *et al.* 1992; Brolin *et al.* 1992; Chodak *et al.* 1992; Bonkhoff *et al.* 1993; Sadi *et al.* 1993; Loda *et al.* 1994; Pertschuk *et al.* 1994; Ruizeveld de Winter *et al.* 1994; Hobisch *et al.* 1995; Pertschuk *et al.* 1995; Hobisch *et al.* 1996; Takeda *et al.* 1996; Magi-Galluzzi *et al.* 1997). Shortly after the sequencing of the AR gene in 1989, monoclonal and polyclonal antibodies were raised against epitopes of the AR protein by generating

small, AR-specific peptides to mimic sequences of the native protein. There are now over 10 commercially available monoclonal or polyclonal anti-AR antibodies in the U.K.

Heterogeneity of AR expression in prostate cancer is well recognised (Sadi *et al.* 1991; Pertschuk *et al.* 1994; Hobisch *et al.* 1995; Pertschuk *et al.* 1995; Takeda *et al.* 1996; Magi-Galluzzi *et al.* 1997) and the receptor remains widely expressed in HRPC. However, there has been only one previous report of AR expression in bone metastases from prostate cancer using IHC (Hobisch *et al.* 1995). The opportunity to examine AR IHC expression in bone metastases is important because bone is the most frequent site of metastases. If AR expression could be related to the presence or absence of AR gene amplification in HRPC samples using IHC, this method may potentially increase its clinical utility.

6.2 Methods

The work was performed in conjunction with the Medical Laboratory Scientific Officers at the UCLH Department of Histopathology, using a robotic slide processor. To ensure that the staining procedure was uniform, all slides, including positive controls (breast tumours expressing AR) and negative controls (normal tonsil lymphoid tissue) were stained in a single run under identical conditions.

The protocol for the AR IHC is listed in full in Appendix 2. Briefly, 5 μ m paraffin section material was mounted on silane-coated slides, prepared and microwaved for antigen retrieval. The slides were then transferred to a Dako Techmate® 500 robotic

immunohistochemistry stainer. This machine performed all the staining steps in the IHC protocol in the following order:

Non-specific tissue peroxidase blocking x 3.

Application and incubation with the primary antibody (Dako anti-human androgen receptor monoclonal mouse antibody AR441, code number M3562).

Application of the secondary biotinylated antibody (rabbit anti-mouse).

Application of the strepavidin conjugated horseradish peroxidase.

Application of the Diaminobenzidine substrate.

H & E slide counterstaining upon completion of the IHC.

All buffer washes and drying steps between the above processes.

Finally, the slides were mounted in DPX medium under a 22x 50 mm coverslip in preparation for analysis.

The semiquantitative Histoscore method was used to assess the degree of immunostaining (Kinsel *et al.* 1989). Briefly, this technique scores all tumour cells into three groups. For each slide, AR nuclear staining was graded as the percentage of nuclei staining with the following pattern; no AR nuclear staining (score 0, blue appearance from the H& E counterstain), some brown peroxidase AR nuclear staining (score 1) and strong brown AR nuclear staining (score 2). The scoring system for the percentage of nuclei staining in each of the 3 ways was as 5% incremental steps (i.e. 20 possible percentage steps for scoring per grade of staining).

Cytoplasmic staining, if seen, was noted but not used for scoring. All tumour areas were reviewed to assess the overall pattern of staining for each slide.

Once the assessment was made, a sum score was produced for each slide. The percentage of cells scoring 1+ staining was multiplied by 1, and the percentage of cells scoring 2+ staining multiplied by 2 and these figures were added to give the sum score. Scores could range from a minimum of zero (100 % negative nuclear staining) to a maximum of 200 (100% 2+ staining multiplied by 2).

To investigate reproducibility and accuracy of scoring, slides were scored by two independent observers. The first observer was myself and the second observer was Dr Ahmet Dogan, Senior Lecturer and Honorary Consultant Histopathologist, University College London. Training in IHC scoring was provided by Dr Dogan and Dr M C Parkinson, Consultant Histopathologist, UCLH Trust. Both observers were blinded to the identification of the slides by a third party who applied additional numbers to each slide, obscuring the original identifying details. This ensured that the previous results of the FISH assessment could not bias the IHC scoring. The first observer also repeated the scoring after a 10-day interval (blinded to the first scoring) to assess intra-observer variability. To further assess variability within a single staining run, duplicates samples (2 or more slides) were included to look for evidence of variation in AR staining patterns from adjacent sections within a single IHC run.

The Histoscore technique was chosen in preference to another common semiquantitative scoring system, the Modified Quick Score (Reiner *et al.* 1990). This system was not used as it combines an assessment of overall staining on low power microscopy (score 1 if weak staining, 2 for moderate staining, 3 for strong staining) with a manual count of 100 tumour cells assessed into 5 groups (<1% staining scores

1, 1-12% scores 2, 12-33% scores 3, 34-66% scores 4, >67% scores 5). As some samples in this study were bone marrow trephine material, containing a majority of non-tumour (normal bone marrow) cells, the low power microscopy component to the Modified Quick Score was not felt to be appropriate. The Modified Quick Score (as with the Histoscore) is frequently used for the assessment of ER and PR staining in breast cancer. The NHS Cancer Screening Program guidelines for Non-operative Diagnostic Procedures and Reporting in Breast Cancer Screening have recommended the Modified Quick Score method as the preferred histological scoring technique (NHS Cancer Screening Program 2001). In this group of samples there is however, a majority of tumour cells for assessment, making the low power microscopy scoring valid (Dr Clive Wells, Consultant Histopathologist, St Bartholomew's Hospital, personal communication). The Histoscore method used in this study has been used in other studies of AR IHC (Pertschuk *et al.* 1995).

In addition to the Histoscore technique, a similar type of analysis was performed using a simplified method to assign a score of 0, 1+ or 2+ to the predominant staining pattern of each slide; no AR staining (0), some AR staining (1+) or strong AR staining (2+). This was termed the Overall score.

Statistics

The Histoscore and Overall scores for each slide were entered into a Microsoft Excel spreadsheet. By manipulating a range of hypothetical 'cut off positive values' for each slide (at which point the IHC Histoscore for the slide was thought to be associated with AR amplification), the data was compared with the results of the FISH studies of the previous chapter. The postulated cut off values for 'positivity' were based on all the observed Histoscores or Overall scores for observer 2 (the experienced observer), from tumours with known AR gene amplification (i.e. all the Histoscore or Overall scores for AR gene amplified tumours scored by observer 2 were assessed). The measurements generated 4 potential results:

A true positive (FISH result positive and IHC value positive).

A true negative (FISH result negative and IHC value negative).

A false negative (FISH result positive and IHC value negative).

A false positive (FISH result negative and IHC value positive).

Two by two contingency tables were constructed, allowing the total number of each of these four outcomes to be counted. Accuracy of an assay is reflected by its sensitivity and specificity in comparison with a standard assay (Wang *et al.* 2001).

From the contingency tables, the sensitivity and specificity of the IHC against FISH was measured. Sensitivity measured the proportion of cases of AR gene amplification correctly identified by the IHC Histoscore technique (true positives / true positives + false negatives). Specificity measured the percentage of cases without AR gene amplification correctly identified by the Histoscore technique (true

negatives / true negatives + false positives). The positive and negative predictive values of the IHC together with the false positive and false negative rates were also calculated in the accepted way (Daly *et al.* 2000). The positive predictive value was the proportion of true cases of AR gene amplification (as measured by FISH) in those IHC cases scored as a positive result. The negative predictive value was the proportion of true cases with no AR gene amplification (as measured by FISH) in IHC cases with a negative result. The false positive rate was the remaining percentage of cases after the positive predictive value was subtracted from 100% and the false negative rate was the remaining percentage of cases after the negative predictive value was subtracted from 100% (Daly *et al.* 2000). These calculations were performed for both observers.

6.3 Results

In total, 30 different specimens were analysed. These consisted of 11 bone marrow trephine samples, 7 TUR samples, 4 TRUS biopsy primary tumour samples, 3 primary radical prostatectomy samples, 2 post-mortem lymph node samples and one supraclavicular fossa lymph node sample. Positive and negative control samples were included to assess the validity of the IHC staining. **Figure 6.1** shows examples of IHC staining patterns found, together with the Histoscores assigned by the observers. The full scoring data set for the 3 sets of observations is listed in **Tables 6.1 - 6.3** below.

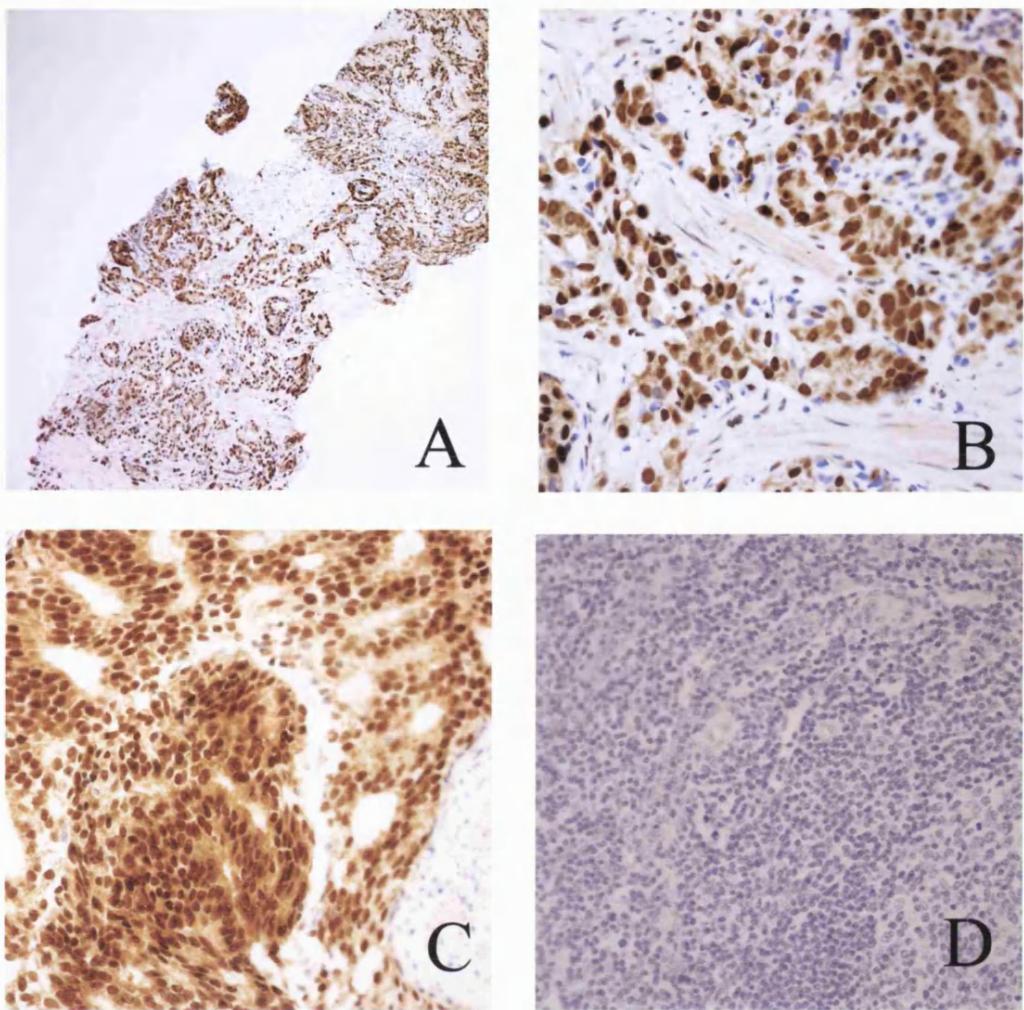


Figure 6.1.

A. Low power view of a TRUS biopsy from patient 16, showing areas of brown AR epithelial immunostaining between the negatively-staining stromal elements (x 100 magnification) **B.** High power view of the same specimen, given a Histoscore of 200 by Observer 2 and 80 by Observer 1 on first scoring and 90 on second scoring 10 days later. AR gene amplification was not present in this primary tumour specimen, and represented a false positive IHC assessment (x 400 magnification). **C.** A TUR section from patient 29 with known AR gene amplification demonstrated on FISH, given Histoscores of 175 by Observer 2 and 160 by Observer 1 (on both scoring

occasions). Despite more pronounced background cytoplasmic staining, there is a small amount of variation in nuclear staining, with some nuclei showing less intense staining than the majority. **D.** The negative control specimen showing no AR IHC brown staining in the nucleus or cytoplasm (Histoscore of 0 by both observers).

Table 6.1.Histoscore and Overall Scores for Observer 1 (RB), 1st assessment.

Patient ID No.	Specimen Type	Amplified on FISH	% 0 Score	% +1 Score	% +2 Score	Total Hscore	Total Overall Score
6	BMT	Y	5	75	20	115	1+
12	BMT	Y	5	20	75	170	2+
15	BMT	Y	0	10	90	190	2+
19	BMT	Y	70	15	15	45	0
22	SCF LN	Y	20	40	40	120	2+
24	PM LN	Y	10	30	60	150	2+
28	TURP	Y	15	60	25	110	1+
<u>29</u>	<u>TURP</u>	<u>Y</u>	<u>10</u>	<u>20</u>	<u>70</u>	<u>160</u>	<u>2+</u>
1	BMT	N	10	30	60	150	2+
3	BMT	N	0	20	80	180	2+
6	TURP	N	25	75	0	75	1+
7	TRUS Bx	N	20	10	70	150	2+
8	RP	N	5	60	35	130	1+
10	BMT	N	5	65	30	125	1+
10	TRUS Bx	N	35	50	15	80	1+
11	BMT	N	5	75	20	115	1+
11	RP	N	60	30	10	50	0
15	TURP	N	50	45	5	55	0
16	BMT	N	10	25	65	155	2+
16	TRUS Bx	N	30	50	20	90	1+
18	BMT	N	10	30	60	150	2+
19	TURP	N	5	70	25	120	1+
20	BMT	N	10	30	60	150	2+
23	PM LN	N	95	5	0	5	0
23	TURP	N	0	60	40	140	1+
24	TRUS Bx	N	25	40	35	110	1+
27	TUR	N	20	65	15	95	1+
27	RP	N	45	50	5	60	1+
AR Neg 1	Tonsil	—	100	0	0	0	0
AR Pos 1	Breast	—	60	30	10	50	0

Legend – see over the page.

Legend for Table 6.1:

AR Neg 1; Negative control 1, AR Pos 1; Positive control 1, BMT; bone marrow trephine, Hscore; Histoscore, ID; identification, N; no, PM LN; post-mortem lymph node, RP; radical prostatectomy, SCF LN; supraclavicular fossa lymph node, TRUS Bx; transrectal ultrasound biopsy, TUR; Transurethral resection, Y; yes.

Table 6.2.Histoscore and Overall Scores for Observer 1 (RB), 2nd assessment (10 days later).

Patient ID No.	Specimen Type	Amplified on FISH	% 0 Score	% +1 Score	% +2 Score	Total Hscore	Total Overall Score
6	BMT	Y	5	70	25	120	1+
12	BMT	Y	10	25	65	155	2+
15	BMT	Y	10	20	70	160	2+
19	BMT	Y	60	20	20	60	0
22	SCF LN	Y	30	50	20	90	1+
24	PM LN	Y	10	50	40	130	1+
28	TURP	Y	15	60	25	110	2+
<u>29</u>	<u>TURP</u>	Y	<u>0</u>	<u>40</u>	<u>60</u>	<u>160</u>	<u>2+</u>
1	BMT	N	10	35	55	145	2+
3	BMT	N	5	15	80	175	2+
6	TURP	N	25	75	0	75	1+
7	TRUS Bx	N	20	20	60	140	2+
8	RP	N	5	60	35	130	1+
10	BMT	N	5	60	35	130	1+
10	TRUS Bx	N	20	75	5	85	1+
11	BMT	N	5	70	25	120	1+
11	RP	N	50	40	10	60	0
15	TURP	N	10	50	40	130	1+
16	BMT	N	0	20	80	180	2+
16	TRUS Bx	N	30	60	10	80	1+
18	BMT	N	10	30	60	150	2+
19	TURP	N	20	50	30	110	1+
20	BMT	N	5	40	55	150	2+
23	PM LN	N	90	10	0	10	0
23	TURP	N	0	70	30	130	1+
24	TRUS Bx	N	20	60	20	100	1+
27	TUR	N	20	60	20	100	1+
27	RP	N	50	35	15	65	0
AR Neg 1	Tonsil	—	100	0	0	0	0
AR1 Pos 1	Breast	—	60	25	15	55	0

Legend – see over the page.

Legend for Table 6.2:

AR Neg 1; Negative control 1, AR Pos 1; Positive control 1, BMT; bone marrow trephine, Hscore; Histoscore, ID; identification, N; no, PM LN; post-mortem lymph node, RP; radical prostatectomy, SCF LN; supraclavicular fossa lymph node, TRUS Bx; transrectal ultrasound biopsy, TUR; Transurethral resection, Y; yes.

Table 6.3.

Histoscore and Overall Scores for Observer 2 (AD).

Patient ID No.	Specimen Type	Amplified on FISH	% 0 Score	% +1 Score	% +2 Score	Total Hscore	Total Overall Score
6	BMT	Y	0	10	90	190	2+
12	BMT	Y	0	20	80	180	2+
15	BMT	Y	0	0	100	200	2+
19	BMT	Y	0	10	90	190	2+
22	SCF LN	Y	0	10	90	190	2+
24	PM LN	Y	0	10	90	190	2+
28	TURP	Y	5	5	90	185	2+
<u>29</u>	<u>TURP</u>	<u>Y</u>	<u>5</u>	<u>15</u>	<u>80</u>	<u>175</u>	<u>2+</u>
1	BMT	N	0	0	100	200	2+
3	BMT	N	0	0	100	200	2+
6	TURP	N	20	60	20	100	1+
7	TRUS Bx	N	0	10	90	190	2+
8	RP	N	100	0	0	0	0
10	BMT	N	0	60	40	140	1+
10	TRUS Bx	N	0	20	80	180	2+
11	BMT	N	0	40	60	160	2+
11	RP	N	10	80	10	100	1+
15	TURP	N	20	70	10	90	1+
16	BMT	N	0	0	100	200	2+
16	TRUS Bx	N	5	10	85	180	2+
18	BMT	N	0	10	90	190	2+
19	TURP	N	5	35	60	155	2+
20	BMT	N	0	5	95	195	2+
23	PM LN	N	90	10	0	10	0
23	TURP	N	0	10	90	190	2+
24	TRUS Bx	N	5	35	60	155	2+
27	TUR	N	10	80	10	100	1+
27	RP	N	30	50	20	90	1+
AR Neg 1	Tonsil	—	100	0	0	0	0
AR Pos 1	Breast	—	10	70	20	110	1+

Legend – see over the page.

Legend for Table 6.3:

AR Neg 1; Negative control 1, AR Pos 1; Positive control 1, BMT; bone marrow trephine, Hscore; Histoscore, ID; identification, N; no, PM LN; post-mortem lymph node, RP; radical prostatectomy, SCF LN; supraclavicular fossa lymph node, TRUS Bx; transrectal ultrasound biopsy, TUR; Transurethral resection, Y; yes.

The results of the sensitivity / specificity analysis for AR IHC are listed in **Table 6.4**. Positive and negative controls were not included in the sensitivity analysis as the samples were not analysed by FISH and were included only to assess the internal validity of the IHC method.

The sensitivity of AR IHC to predict AR gene amplification using either the Histoscore or Overall method was between 33-67%, depending on the cut-off value. The lowest sensitivities were for observer 1, whose Histoscore sensitivity ranged between 33-38% and whose Overall score sensitivity was between 33-67%. For Observer 2, Histoscore sensitivity ranged from 38 to 67% and the Overall score sensitivity was 67%. In general, sensitivity for Observer 2 was slightly better than Observer 1 (see **Table 6.4**). The positive predictive values ranged between 27-36% for the Histoscore method and 29-38% for the Overall score method. False positive rates were consistently in the 60 to 70% ranges for both observers and for both scoring methods.

The specificity of the Histoscore method (the ability to correctly predict tumours with no AR gene amplification) ranged between 52-65% (i.e. a greater success at identifying true negative cases with no AR gene amplification than for true positive cases where AR gene amplification was present). For the Overall score method the specificity was between 37% and 67%. In general, the specificity for Observer 1 was slightly better than Observer 2 (see **Table 6.4**). In conjunction with these sensitivity results, false negative rates were in the range of 20-30% for both observers and both scoring systems. The negative predictive values were 67-79 % for the Histoscore method and 62-78% for the Overall score method.

Table 6.4

Sensitivity and specificity analysis for AR immunohistochemistry as a predictor of AR gene amplification by FISH.

Observer	RB1	RB2	AD
	Cut off Hscore 200	Cut off Hscore 200	Cut off Hscore 200
Sensitivity	33%	33%	38%
Specificity	67%	67%	65%
PPV	29%	29%	30%
NPV	71%	71%	72%
FP Rate	71%	71%	70%
FN Rate	29%	29%	28%
	Cut off Hscore 190	Cut off Hscore 190	Cut off Hscore 190
Sensitivity	33%	33%	54%
Specificity	67%	67%	55%
PPV	29%	29%	33%
NPV	71%	71%	75%
FP Rate	71%	71%	67%
FN Rate	29%	29%	25%
	Cut off Hscore 180	Cut off Hscore 180	Cut off Hscore 180
Sensitivity	38%	33%	63%
Specificity	65%	65%	52%
PPV	30%	28%	34%
NPV	72%	71%	78%
FP Rate	70%	72%	66%
FN Rate	28%	29%	22%

Legend:

AD; Dr Ahmet Dogan, FN; false negative, FP; false positive, Hscore; Histoscore, Oscore; Overall score, PPV; positive predictive value, NPV; negative predictive value, RB1 & RB2; Richard Brown, 2 sets of blinded observations, 10 days apart.

Table 6.4 (Continued)

Sensitivity and specificity analysis for AR immunohistochemistry as a predictor of AR gene amplification by FISH.

Observer	RB1	RB2	AD
	Cut off Hscore 175	Cut off Hscore 175	Cut off Hscore 175
Sensitivity	38%	33%	67%
Specificity	65%	63%	52%
PPV	30%	27%	36%
NPV	72%	70%	79%
FP Rate	70%	73%	64%
FN Rate	28%	30%	21%

	Cut off Oscore 2+	Cut off Oscore 2+	Cut off Oscore 2+
Sensitivity	33%	50%	67%
Specificity	67%	57%	47%
PPV	29%	32%	33%
NPV	71%	74%	78%
FP Rate	71%	68%	68%
FN Rate	29%	26%	26%

	Cut off Oscore >/=1+	Cut off Oscore >/=1+	Cut off Oscore >/=1+
Sensitivity	67%	63%	67%
Specificity	49%	38%	37%
PPV	38%	29%	30%
NPV	76%	72%	73%
FP Rate	62%	71%	71%
FN Rate	24%	28%	28%

Legend:

AD; Dr Ahmet Dogan, FN; false negative, FP; false positive, Hscore; Histoscore, Oscore; Overall score, PPV; positive predictive value, NPV; negative predictive value, RB1 & RB2; Richard Brown, 2 sets of blinded observations, 10 days apart.

Twelve patient samples and the 2 control samples were also duplicated in the IHC run to assess staining variation within the run. This represented 23 additional slides. There was sample agreement to within 10% in 8 out of the 14 duplicate cases (57%) using either the Histoscore or Overall score method. Of the 6 cases where there was discordance between samples, in 3 cases this was marked (Histoscores of 150 and 80, 95 and 15, 120 and 60).

Intra-observer variation by Observer 1 (RB) over a 10-day period lay between 0% (10 cases) and 50% (1 case). The median difference was 5% and the mean 10% (see **Table 6.5** below).

Table 6.5.

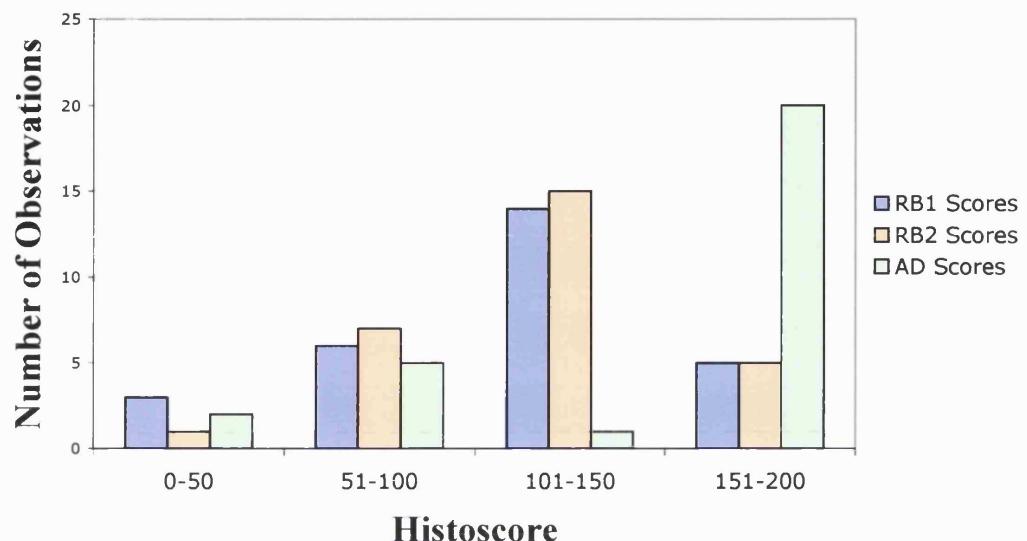
Intra observer variation in Histoscore values over a 10-day period.

Patient ID Number	Maximum Intra-Observer Difference
1	5%
3	5%
6	0%
6	5%
7	10%
8	0%
10	5%
10	25%
11	0%
11	10%
12	5%
15	20%
15	50%
16	15%
16	10%
18	0%
19	10%
19	20%
20	10%
22	20%
23	5%
23	10%
24	20%
24	20%
27	5%
27	15%
28	0%
29	20%
AR Negative Control 1	0%
AR Positive Control 1	0%

Heterogeneity of AR expression was seen in almost all samples. Four samples from Observer 2 were scored with a maximum Histoscore of 200, indicating no significant variation and a very intense staining pattern. Observer 1 did not score any samples on either of the two assessments with a Histoscore maximum of 200. Observer 2 scored only 1 sample with a Histoscore of 0 and Observer 2 did not score any samples as negative (not counting the negative control sample). For each set of observations on the 28 samples scored (not including the positive and negative controls), the distribution is shown graphically in **Figure 6.2** to demonstrate how the two observers varied. Observer 2 scored a greater percentage of samples with a Histoscore of 151-220 and Observer 1 a greater percentage of 101-150 scores.

Figure 6.2.

Intra- and Inter- Observer Variations in Histoscore Ranges



6.4 Discussion

The results of AR IHC as a method for predicting AR gene amplification as demonstrated by FISH are disappointing. The maximum sensitivity of the test was 67% (at a Histoscore cut-off value for positivity of 175), indicating that at best, the method could predict 2 out of 3 cases of AR gene amplification. This level of sensitivity was however associated with a positive predictive value of only 36% and a false positive rate of 64%.

For AR IHC to be clinically useful in predicting AR gene amplification, the characteristic of the test of greatest relevance to the management of individual patients is the positive predictive value (PPV), i.e. the probability that AR gene amplification is present when the test is positive. In this study, the best PPV was only 36% (Observer 2, AD, using a Histoscore cut off for positivity of 175). This suggests that if IHC were used to predict AR gene amplification, in 2 out of the 3 cases a positive test results would not be associated with AR gene amplification. This is an unacceptably high figure and the IHC test cannot be recommended as a substitute for FISH in predicting the presence of AR gene amplification.

The specificity of the IHC methods was greater than the sensitivity, with figures in the range of 60-70%. Identifying patients who do not carry the AR gene amplification (rather than correctly identifying the true positive cases) may also help to classify patients for treatment, but false negative results still occurred in approximately one quarter of cases. Taken in combination with the sensitivity

analysis this figure is also too high to recommend the use of AR IHC as a substitute for FISH in the demonstration of AR gene amplification.

These results should be examined in relation to concordance data for *c-erbB2* gene amplification detected by FISH, and *c-erbB2* protein overexpression detected by IHC. The optimum method for detecting *c-erbB2* status in breast cancer has been hotly debated. Some authors have regarded FISH as optimum methodology (Bartlett *et al.* 2001; Gancberg *et al.* 2001) while others have felt that the sensitivity and specificity of IHC is sufficient to allow the determination of *c-erbB2* in most cases (Jacob *et al.* 1999; Couturier *et al.* 2000). Different studies have suggested that IHC for *c-erbB-2* lacks sensitivity and specificity, and that IHC is subject to considerable interobserver variability (Kay *et al.* 1994; Press *et al.* 1994). However, concordance of *c-erbB-2* status for both methods is now generally regarded as good for tumours with no expression of *c-erbB2* (no amplification demonstrated by FISH and no or weakly positive 1+ IHC staining) and for amplified tumours (amplification demonstrated by FISH and strongly positive 3+ IHC staining). Figures in the range of 80-95% have generally been reported (Couturier *et al.* 2000; Schnitt *et al.* 2001; Thomson *et al.* 2001; Wang *et al.* 2001), sufficient to allow the use of IHC to demonstrate *c-erbB-2* overexpression. Lower sensitivity and specificity have been reported in tumours that stain with moderate intensity (2+) using IHC (Hoang *et al.* 2000; Tsuda *et al.* 2001; Perez *et al.* 2002). False positive results (positive using IHC and negative using FISH) have been the most frequently reported problem contributing to this observation (Couturier *et al.* 2000; Tsuda *et al.* 2001).

Both FISH and IHC are now widely used in determining c-erbB-2 status in breast cancer with many institutions now using IHC as a screening step followed by FISH in selected cases (Schnitt *et al.* 2001).

There are a number of possible reasons why AR IHC failed to accurately predict AR gene amplification. It is known that the IHC horseradish peroxidase step (converting the diaminobenzidene substrate into the brown stain), is a non-linear reaction (Miller 2000). Consequently the intensity of staining is not directly proportional to the amount of antigen detected by the primary antibody. The use of a non-linear process is a weakness in the methodology if it is to be used for quantitation. However, this limitation has not prevented the IHC method being used to assess cerb-B2 overexpression in breast cancer (Lebeau *et al.* 2001).

It is possible that the relative levels of expression of c-erbB-2 protein in breast cancer are higher than those of AR protein expression in HRPC. Consequently, c-erbB-2 protein may be assessable by the semi-quantitative IHC method, resulting in a better correlation of c-erbB-2 protein levels with gene amplification. In cases of AR gene amplification in HRPC, expression of AR protein cDNA is more than 2 times higher than that in non-amplified HRPC samples (Linja *et al.* 2001). However, non-amplified samples contained a 6-fold median increase in levels of AR cDNA expression compared with primary tumours or cases of BPH. The cerbB-2 protein expression associated with c-erbB-2 gene amplification is known to be significantly elevated (Reese *et al.* 1997). Studies looking at c-erbB-2 protein using Western Blot or ELISA techniques have been performed (Slamon *et al.* 1989; Borg *et al.* 1990; Molina *et al.* 1992; Giai *et al.* 1994; Eissa *et al.* 1997) but direct comparison of the

levels of AR and c-erbB-2 proteins is difficult and not readily available. In a recent study by Savinainen *et al.*, breast tumours with *c-erbB-2* amplification expressed, on average, approximately 20 times higher mRNA levels than prostate (including HRPC) or breast tumours without gene amplification (Savinainen *et al.* 2002).

Marked heterogeneity of AR staining within prostate tumours is also well recognised. The biological significance of such observations is not clear in relation to AR gene amplification. In this study, for Observer 1, the percentage of negative Histoscores in tumours known to have AR gene amplification by FISH was between 0 to 70% and for Observer 2 was between 20-70%, depending on the cut-off point. It is possible that androgen receptor gene amplification is not always associated with AR protein production, but the available evidence does not support this being a common finding (Koivisto *et al.* 1997; Linja *et al.* 2001).

Negative Histoscores in cases of AR gene amplification could also occur because some tumours do not contain the epitope that the antibody detects, or that the method failed for technical reasons. Optimal results using IHC depend on the quality of the tissue examined (Gancberg *et al.* 2001). All bone marrow samples were processed in the same laboratory (the UK reference laboratory for oestrogen and progesterone receptor IHC), but a large proportion of primary tumour samples were obtained from different laboratories, making it difficult to standardise fixation and processing. Unless all tissue samples can be processed in a similar fashion, some degree of variation will occur in the analysis of gene expression by IHC (Bartlett *et al.* 2001).

It has been shown here, and in the study by Hobisch, (1995), that AR staining can be demonstrated by IHC in decalcified bone samples. However it is not known if decalcification affects AR IHC expression. Proteins are generally more sensitive to fixation techniques than DNA (Miller 2000), which remains relatively stable after fixation and decalcification. This lack of standardization may have had an impact on the ability to reliably demonstrate AR IHC staining.

The intra-observer variation between the 2 assessments by Observer 1 was generally acceptable (median difference 5% and mean difference 10%). However 8/30 (27%) of the repeat observations were equal to or greater than 20%. It is possible that further improvement would come with greater experience, but the overall disappointing performance of AR IHC to predict the FISH results was not due to intra-observer variation.

Inter-observer variation between Observer 1 and Observer 2 (an experienced Histopathologist) was to be expected, and was most marked in the differences between Histoscore values of 100-200 (i.e. positive results with some degree of staining). Compared with Observer 2, Observer 1 tended to score positive staining at a lower overall value (**Figure 6.2**). This may also have been improved with further training, but was not the reason for failure of the method to be useful in predicting AR gene amplification.

The simplified Overall score method used in this study did not offer any advantage over the Histoscore method, with little difference in the best performance for either observer.

This study confirms the findings of the only previous study on AR IHC expression in bone metastases from prostate cancer (Hobisch *et al.* 1995). In this study, 18 bone metastases from advanced prostate cancer (17/18 patients had previous endocrine therapy) were assessed using a polyclonal antibody. AR IHC positivity was found in all 18 cases. In the current study, a mouse monoclonal antibody was used for AR IHC and a degree of AR positivity (i.e. a Histoscore score greater than zero) was found in all 12 cases by Observer 1 and in 11/12 cases by observer 2. These observations add further support to the theory that AR expression remains important in HRPC.

In summary, this work has shown that AR IHC, using a commercially available monoclonal anti-AR antibody, was unable to successfully predict the presence of AR gene amplification as detected by FISH and cannot be recommended as an alternative methodology. The recent report of an investigational in situ method to evaluate gene copy number using routine light microscopy (chromogenic in situ hybridisation, CISH) may hold future promise for identifying AR gene amplification in diagnostic pathology laboratories (Tanner *et al.* 2000).

Chapter 7:
Summary and Future Directions

7.1 Summary

The work in this thesis has achieved a number of the aims set out at the end of the introductory section (pages 75-77). It was possible to establish a tissue resource of bone and bone marrow biopsies from patients with HRPC, together with matching primary tumour samples, where they were available. Such a resource is not widely available for the study of distant metastases from HRPC.

In this series, bone marrow trephine biopsies, but not bone marrow aspirates, yielded tumour material. Aspirates, which would have been easier to work with, failed to show any malignant cells. The work has allowed an evaluation of a method for targeting bone metastases (demonstrated on diagnostic 99m Tc-bone scans) and assesses the sampling yield of this approach (Brown *et al.* 2002). Bone metastases from men with HRPC are not routinely biopsied, and there is a paucity of information available on the success rate, or optimum methods.

This work has also shown that the necessary decalcification, required to work with bone material, can be carried out using a brief 5% formic acid digestion, preserving DNA for molecular techniques such as FISH (Brown *et al.* 2002). There are publications that indicate that molecular studies are compromised by some of the methods need to decalcify bone biopsies for sectioning.

The studies here have also shown that the prevalence of AR gene amplification can be measured in bone metastases from HRPC. Other workers have reported early results suggesting that identification of AR gene amplification may enable patients to

be selected for second line hormone treatments (Palmberg *et al.* 2000, see below). If this proves to be the case, the clinical utility of bone marrow trephine sampling and FISH studies may be sufficient to justify its use in the clinic.

Immunohistochemistry did not prove to be a satisfactory method of predicting which HRPC tumours demonstrated AR gene amplification and cannot be recommended as an alternative to FISH.

7.2 Future Directions

The recent, prospective, pilot study from the group who described AR gene amplification (Palmberg *et al.* 2000), demonstrated a more favourable response to second line maximum androgen blockade (MAB) in those patients with AR gene amplification. This work now provides further areas to explore in the study of AR gene amplification. As stated by the authors, most patients experience progression at sites of distant metastases and it should be these sites that are analysed for AR gene amplification. This has usually not been possible (Koivisto *et al.* 1995; Visakorpi *et al.* 1995; Koivisto *et al.* 1997; Miyoshi *et al.* 2000; Palmberg *et al.* 2000; Ware *et al.* 2000; Edwards *et al.* 2001; Haapala *et al.* 2001; Linja *et al.* 2001) or if it has been done it has been performed on post-mortem material (Bubendorf *et al.* 1999; Kaltz-Wittmer *et al.* 2000). In their recent study, Palmberg *et al.*, recommended performing FISH on bone marrow metastases from carcinoma of the prostate and the work in this thesis has shown for the first time that this can be achieved (Palmberg *et al.* 2000).

The next logical study to perform would be a further prospective study seeking to confirm the findings of the pilot by Palmberg *et al.* in patients with progression of HRPC in bone, looking at response to second-line MAB therapy in terms of time to progression, response rates to treatment and overall survival. Such a study would require two bone marrow trephine samples, at initial disease progression to define patients showing AR gene amplification, and possibly at a further point in the course of treatment in an attempt to define if the natural history of AR gene amplification changes as a response to therapy. Such a study would require the co-operation and a significant degree of goodwill from patients (as the work presented here did). Bone marrow trephine biopsy has not been a regular part of the work up of patients with HRPC. Other patients, such as those with haematological malignancies, undergo repeated bone marrow biopsies throughout the course of their treatment (both curative and palliative). Although HRPC therapy would be palliative in nature, it may be possible for patients to accept bone marrow trephine sampling if therapy choices result from it.

If the results confirm that patients with androgen receptor amplification form a distinct subgroup of patients, then there are a number of alternative treatments that might be tested. Maximum androgen blockade is not usually given in the UK as the first treatment for relapsed prostate cancer, but at progression following first-line androgen withdrawal therapy MAB is often prescribed. Separation of patients into those likely to respond to additional antiandrogens would allow patients to be targeted with more appropriate therapy. Patients not demonstrating AR gene amplification could be treated in other ways and could be spared additional

antiandrogen therapy and the side effects of these agents. Potentially this approach may also result in cost savings.

The AR protein remains a challenging target in other directions for further therapy. New pharmacological interventions at the level of AR-ligand interactions, together with agents that affect N- and C-terminal interactions and co-activator and co-repressor interactions, are waiting to be developed (Gelmann 2002). As AR protein expression remains a feature in HRPC, (Ruizeveld de Winter *et al.* 1994; Hobisch *et al.* 1995; Pertschuk *et al.* 1995; Takeda *et al.* 1996; Koivisto *et al.* 1997; Magi-Galluzzi *et al.* 1997; Koivisto *et al.* 1998; Linja *et al.* 2001) the entire androgen pathway remains a therapeutic target for new, novel treatments. Amplification of the AR gene leading to higher levels of the protein expression makes treatment targeted at the receptor complex attractive in these patients. Interest is now being shown in the ansamycin antibiotic compound geldanamycin, derived from *Streptomyces hygroscopicus* (Kuduk *et al.* 2000; Solit *et al.* 2002; Vanaja *et al.* 2002). This agent is a Heat Shock Protein 90 (Hsp90) inhibitor, which when bound to the Hsp90-AR receptor heterocomplex (see Chapter 1), results in loss of steroid receptor activity and degradation of the AR complex. A cell line and xenograft study has been published on this agent (Solit *et al.* 2002). The initial data suggests that at tolerable doses, geldanamycin reduces AR expression and inhibits prostate tumour growth in mice (Solit *et al.* 2002). If levels of AR expression are elevated in AR gene amplified HRPC (Linja *et al.* 2001), there is a rationale for studying geldanamycin or geldanamycin-testosterone hybrids (Kuduk *et al.* 2000) in HRPC model systems, such as the recently described cell line containing an AR gene amplification (Linja *et al.* 2001).

In HRPC AR gene amplified tumours, the other area of future therapy emerging that may offer promise is antisense technology. A number of preclinical studies using oligodeoxynucleotides (ODNs) on prostate cell lines and tumour xenografts have been reported (Devi 2002; Eder *et al.* 2002). Early clinical trials using ODNs are now also underway (see Devi 2002). Eder *et al.* have reported two studies using ODNs directed against the AR, but not using tumours known to contain the AR gene amplification (Eder *et al.* 2002). The authors demonstrated that subcutaneous infusion pumps delivering an ODN directed against the AR CAG repeat, resulted in significant tumour growth inhibition in male nude mice carrying a human LNCaP cell xenograft (in comparison to untreated control animals). A cell line containing AR gene amplification (Linja *et al.* 2001) would be an attractive modelling system for studies on the effects of ODNs in AR gene amplified cells before clinical studies could be undertaken.

References

Alers J C, Krijtenberg P-J, Vissers K J, Bosman F T, van der Kwast T H, van Dekken H. Interphase cytogenetics of prostatic adenocarcinoma and precursor lesions: analysis of 25 radical prostatectomies and 17 adjacent prostatic intraepithelial neoplasias. *Genes Chromosomes Cancer* 1995;12:241-250.

Alers J C, Krijtenberg P-J, Vissers K J, Krishnadath S K, Bosman F T, van Dekken H. Interphase *in situ* hybridization to disaggregated and intact tissue specimens of prostatic adenocarcinomas. *Histochem Cell Biol* 1995;104:479-486.

Alers J C, Krijtenburg P J, Rosenberg C, Hop W C, Verkerk A M, Schroder F H, van der Kwast T H, Bosman F T, van Dekken H. Interphase cytogenetics of prostatic tumor progression: specific chromosomal abnormalities are involved in metastasis to the bone. *Lab Invest* 1997;77:437-48.

Alers J C, Krijtenberg P-J, Vissers K J, van Dekken H. Effect of bone decalcification procedures on DNA *in situ* hybridization and comparative genomic hybridization: EDTA is highly preferable to a routinely used acid decalcifier. *J Histochem Cytochem* 1999;47:703-709.

Alers JC. Krijtenburg PJ. Vis AN. Hoedemaeker RF. Wildhagen MF. Hop WC. van Der Kwast TT. Schroder FH. Tanke HJ. van Dekken H. Molecular cytogenetic analysis of prostatic adenocarcinomas from screening studies: early cancers may contain aggressive genetic features. *Am J Pathol* 2001;158:399-406.

Algra P R, Bloem J L, Tissing H, Falke T H M, Arndt J W, Verboom L J. Detection of vertebral metastases: comparison between MR imaging and bone scintigraphy. *Radiographics* 1991;11:219-232.

Alyea E P, Rundles R W. Bone marrow studies in carcinoma of the prostate. *J Urol* 1949;62:332-339.

Anzick S L, Kononen J, Walker R L, Azorsa D O, Tanner M M, Guan X Y, Sauter G, Kallioniemi O P, Trent J M, Meltzer P S. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997;277:965-968.

Arnold J T, Isaac J T. Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cell's fault. *Endocr Relat Cancer* 2002;9:61-73.

Bartlett J M S, Going J J, Mallon E A, Watters A D, Reeves J R, Stanton P, Richmond J, Donald B, Ferrier R, Cooke T G. Evaluating *HER2* amplification and overexpression in breast cancer. *J Pathol* 2001;195:422-428.

Bashirelahi N, Young J D, Sidh S M, Sanefuji H. Androgen, oestrogen, and progesterone and their distribution in epithelial cells and stromal cells of the human prostate. In: Steroid receptors, metabolism and prostate cancer. Schroder FH, de Voogt HJ (Ed). Amsterdam, Excerpta Medica 1979.

Bearden J D, Ratkin G A, Coltman C A. Comparison of the diagnostic value of bone marrow biopsy and bone marrow aspiration in neoplastic disease. *J Clin Pathol* 1974;27:738-740.

Beato M, Sanchez-Pacheco A. Interaction of steroid hormone receptors with the transcription initiation complex. *Endocr Rev* 1996;17:587-609.

Beheshti B, Vukovic B, Marrano P, Squire J A, Park P C. Resolution of genotypic heterogeneity in prostate tumors using polymerase chain reaction and comparative genomic hybridization on microdissected carcinoma and prostatic intraepithelial neoplasia foci. *Cancer Genet Cytogenet* 2002;137:15-22.

Beilin J, Harewood L, Frydenberg M, Mameghan H, Martyres R F, Farish S J, Yue C, Deam D R, Byron K A, Zajac J D. A case-control study of the androgen receptor gene CAG repeat polymorphism in Australian prostate carcinoma subjects. *Cancer* 2001;92:941-949.

Belchetz P. Male hypogonadism. In: Clinical Endocrinology. Grossman A (Ed). Oxford, Blackwell Science 1998.

Berrevoets C A, Doesburg P, Steketee K, Trapman J, Brinkmann A O. Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor 2). *Mol Endocrinol* 1998;12:1172-1183.

Blok L J, de Ruiter P E, Brinkmann A O. Forskolin-induced dephosphorylation of the androgen receptor impairs ligand binding. *Biochemistry* 1998;37:3850-3857.

Bonkhoff H, Stein U, Remberger K. Androgen receptor status in endocrine-paracrine cell types of the normal, hyperplastic, and neoplastic human prostate. *Virchows Archiv* 1993;423:291-294.

Borg A, Tandon A T, Sigurdsson H, Clark G M, Ferno M, Fuqua S A W, Killander D, McGuire W L. HER-2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res* 1990;50:4332-4337.

Bosland M S. The role of steroid hormones in prostate carcinogenesis. *J Natl Cancer Inst Monogr* 2000;27:39-66.

Bova G S, Isaacs W B. Review of allelic loss and gain in prostate cancer. *World J Urol* 1996;14:338-346.

Brinkmann A O, Blok L J, de Ruiter P E, Doesburg P, Steketee K, Berrevoets C A, Trapman J. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* 1999;69:307-313.

Brolin J, Lowhagen T, Skoog L. Immunocytochemical detection of the androgen receptor in fine needle aspirates from benign and malignant human prostate. *Cytopathology* 1992;3:351-357.

Brolin J, Skoog L, Ekman P. Immunohistochemistry and biochemistry in detection of androgen, progesterone, and estrogen receptors in benign and malignant human prostatic tissue. *Prostate* 1992;20:281-295.

Brown C J, Goss S J, Lubahn D B, Joseph D R, Wilson E M, French F S, Willard H F. Androgen receptor locus on the human X chromosome: regional localization to

Xq11-12 and description of a DNA polymorphism. *Am J Hum Genet* 1989;44:264-269.

Brown T R. Human androgen insensitivity syndrome. *J Androl* 1995;16:299-303.

Brown R S D, Dogan A, Ell P J, Payne H A, Masters J R W, Harland S J. The comparative values of bone marrow aspirate and trephine for obtaining bone scan-targeted metastases from hormone refractory prostate cancer. *Prostate Cancer Prostatic Dis* 2002;5:144-151.

Brown R S D, Edwards J, Bartlett J W, Jones C, Dogan A. Routine acid decalcification of bone marrow samples can preserve DNA for FISH and CGH studies in metastatic prostate cancer. *J Histochem Cytochem* 2002;50:113-115.

Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser T C, Willi N, Mihatsch M J, Sauter G, Kallioniemi O P. Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. *Cancer Res* 1999;59:803-806.

Bubulya A, Wise S C, Shen X Q, Burmeister L A, Shemshedini L. c-Jun can mediate androgen receptor-induced transactivation. *J Biol Chem* 1996;271:24583-24589.

Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee H J, Wang C, Mizokami A. Androgen receptor: an overview. *Crit Rev Eukaryot Gene Expr* 1995;5:97-125.

Chang C S, Kokontis J, Liao S T. Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science* 1988;240:324-326.

Cher M L, Bova G S, Moore D H, Small E J, Carroll P R, Pin S S, Epstein J I, Isaacs W B, Jensen R H. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res* 1996;56:3091-3102.

Chodak G W, Kranc D M, Puy L A, Takeda H, Johnson K, Chang C. Nuclear localization of androgen receptor in heterogeneous samples of normal, hyperplastic and neoplastic human prostate. *J Urol* 1992;147:798-803.

Chu L W, Pettaway C A, Liang J C. Genetic abnormalities specifically associated with varying metastatic potential of prostate cancer cell lines as detected by comparative genomic hybridization. *Cancer Genet Cytogenet* 2001;127:161-167.

Chu L W, Troncoso P, Johnston D A, Liang J C. Genetic markers useful for distinguishing between organ-confined and locally advanced prostate cancer. *Genes Chromosomes Cancer* 2003;36:303-312.

Chua D T, Ackermann A, Veenema R J. Bone marrow biopsy in patients with carcinoma of the prostate. *J Urol* 1969;102:602-606.

Clifton J A, Philipp R J, Ludovic E, Fowler W M. Bone marrow and carcinoma of the prostate. *Am J Med Sci* 1952;224:121-130.

Couturier J, Vincent-Salomon A, Nicolas A, Beuzeboc P, Mouret E, Zafrani B, Sastre-Garau X. Strong correlation between results of fluorescent *in situ* hybridization and immunohistochemistry for the assessment of ERBB2 (HER-2/neu) gene status in breast carcinoma. *Mod Pathol* 2000;13:1238-1243.

Craft N, Shostak Y, Carey M, Sawyers C L. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signalling by the HER-2/neu tyrosine kinase. *Nature Med* 1999;5:280-285.

Crisp J. Random bone marrow biopsy in the staging of carcinoma of the prostate. *Br J Urol* 1976;48:265-267.

Culig Z, Hobisch A, Cronauer M V, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* 1994;54:5474-5478.

Culig Z, Hobisch A, Hittmair A, Cronauer M V, Radmayr C, Zhang J, Bartsch G, Klocker H. Synergistic activation of androgen receptor by androgen and luteinizing hormone-releasing hormone in prostatic carcinoma cells. *Prostate* 1997;32:106-114.

Cunha G R, Chung W K. Stromal-epithelial interactions-I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. *J Steroid Biochem* 1981;14:1317-1321.

Cunha G R, Donjacour A A, Cooke P S, Mee S, Bigbsy R M, Higgins S J, Sugimura Y. The endocrinology and developmental biology of the prostate. *Endocr Rev* 1987;8:338-362.

Daly L E, Bourke G J. Bias and measurement errors. In: *Interpretation and use of medical statistics*. Daly L E, Bourke G J (Ed). Oxford, Blackwell Science 2000.

de Ruiter P E, Teuwen R, Trapman J, Dijkema R, Brinkmann A O. Synergism between androgens and protein kinase-C on androgen-regulated gene expression. *Mol Cell Endocrinol* 1995;110:R1-R6.

de Voogt H J, Rao B R, Geldof A A, Gooren L J G, Bouman F G. Androgen action blockade does not result in reduction in size but changes the histology of the normal human prostate. *Prostate* 1987;11:305-313.

de Voogt H J, Studer U, Schroder F H, Klijn J G, de Pauw M, Sylvester R. Maximum androgen blockade using LHRH agonist buserelin in combination with short term (two weeks) or long term (continuous) cyproterone acetate is not superior to standard androgen deprivation in the treatment of advanced prostate cancer. Final analysis of EORTC GU group trial 30843. *Eur Urol* 1998;33:152-158.

Deguchi T, Yang M, Ehara E, Ito S, Nishino Y, Takahashi Y, Ito Y, Shimokawa K, Tanaka T, Imaeda T. Detection of micrometastatic prostate cancer cells in the bone marrow of patients with prostate cancer. *Br J Cancer* 1997;75:634-638.

Devi G R. Prostate cancer: status of current treatments and emerging antisense-based therapies. *Curr Opin Mol Ther* 2002;4:138-148.

Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351:1451-1467.

Eder I E, Culig Z, Ramoner R, Thurnher M, Putz T, Nessler-Menardi C, Tiefenthaler M, Bartsch G, Klocker H. Inhibition of LNCaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther* 2000;7:997-1007.

Eder I E, Culig Z, Putz T, Nessler-Menardi C, Bartsch G, Klocker H. Molecular biology of the androgen receptor: from molecular understanding to the clinic. *Eur Urol* 2001;40:241-251.

Eder I E, Hoffman J, Rogatsch H, Schafer G, Zopf D, Bartsch G, Klocker H. Inhibition of LNCaP prostate tumour growth in vivo by an antisense oligonucleotide directed against the human androgen receptor. *Cancer Gene Ther* 2002;9:117-125.

Edwards J, Krishna N S, Mukherjee R, Watters A D, Underwood M A, Bartlett J M S. Amplification of the androgen receptor may not explain development of androgen independent prostate cancer. *Br J Urol* 2001;88:633-637.

Eissa S, Khalifa A, El-Gharib A, Salah N, Mohamed M K. Multivariate analysis of DNA ploidy, p53, c-erbB-2 proteins, EGFR, and steroid hormone receptors for short term prognosis in breast cancer. *Anticancer Res* 1997;17:3091-3098.

Elo J P, Visakorpi T. Molecular genetics of prostate cancer. *Ann Med* 2001;33:130-141.

Faber P W, Kuiper G G, van Rooij H C, van der Korput J A, Brinkmann A O, Trapman J. The N-terminal domain of the human androgen receptor is encoded by one, large exon. *Mol Cell Endocrinol* 1989;61:257-262.

Fang S, Anderson K M, Liao S. Receptor proteins for androgens: on the role of specific proteins in selective retention of 17-beta-hydroxy-alpha-androstan-3-one by rat ventral prostate in vivo and in vitro. *J Biol Chem* 1969;244:6584-6595.

Fletcher J A. DNA in situ hybridization as an adjunct to tumour diagnosis. *Am J Clin Pathol* 1999;112 (Suppl 1):S11-S18.

Galasko C S B. The anatomy and pathways of skeletal metastases. In: Bone metastases. Weiss L, Gilbert HA (Ed). Boston, G K Hall 1981.

Gall G J, Pardue M L. Formation of RNA-DNA hybrid molecules in cytogenetical preparations. Proc Natl Acad Sci USA 1969;63:378-383.

Gancberg D, Lespagnard L, Rouas G, Paesmans M, Piccart M, Di Leo A, Nogaret J-M, Hertens D, Verhest A, Larsimont D. Sensitivity of HER-2/neu antibodies in archival tissue samples of invasive breast cancer: correlation with oncogene amplification in 160 cases. Am J Clin Pathol 2001;113:675-682.

Gelmann E P. Molecular biology of the androgen receptor. J Clin Oncol 2002;20:3001-3015.

Gerber H P, Seipel K, Georgiev O, Hug M, Rusconi S. Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science 1994;263:808-811.

Giai M, Roagna R, Ponzone R, De Bertoli M, Dati C, Sismondi P. Prognostic and predictive relevance of c-erbB-2 and ras expression in node positive and node negative breast cancer. Anticancer Res 1994;14:1441-1450.

Gottlicher M, Heck S, Herrlich P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med 1998;76:480-489.

Gottlieb B, Beitel L K, Lumbroso R, Pinsky L, Trifiro M. Update of the androgen receptor gene mutations database. Hum Mutat 1999;14:103-114.

Gozzetti A, Le Beau M M. Fluorescence in situ hybridization: uses and limitations. Semin Hematol 2000;37:320-333.

Gregory C W, He B, Johnson Jr. R T, Ford O H, Mohler J L, French F S, Wilson E M. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 2001;61:4315-4319.

Gregory C W, Johnson Jr. R T, Mohler J L, French F S, Wilson E M. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res* 2001;61:2892-2898.

Grossmann M E, Huang H, Tindall D J. Androgen receptor signalling in androgen-refractory prostate cancer. *J Nat Cancer Inst* 2001;93:1687-1697.

Haapala K, Hyytinen E R, Roiha M, Laurila M, Rantala I, Helin H J, Koivisto P A. Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchietomy and bicalutamide. *Lab Invest* 2001;81:1647-1651.

Haapala K, Rokman A, Palmberg C, Hyytinen E R, Laurila M, Tammela T L, Koivisto P A. Chromosomal changes in locally recurrent, hormone-refractory prostate carcinomas by karyotyping and comparative genomic hybridization. *Cancer Genet Cytogenet* 2001;131:74-78.

Haddock G, Gray H W, McKillop J H, Bessent R G, Kirk D. ^{99m}Tc-Nanocolloid bone marrow scintigraphy in prostate cancer. *Br J Urol* 1989;63:497-502.

Helenius M A, Saramaki O R, Linja M J, Tammela T L, Visakorpi T. Amplification of urokinase gene in prostate cancer. *Cancer Res* 2001;61:5340-5344.

Heller A, Chudoba I, Bleck C, Senger G, Claussen U, Liehr T. Microdissection based comparative genomic hybridization analysis (micro-CGH) of secondary acute myelogenous leukemias. *Int J Oncol* 2000;16:461-468.

Hiort O, Holterhus P M, Nitsche E M. Physiology and pathophysiology of androgen action. *Baillieres Clin Endocrinol Metab* 1998;12:115-132.

Hoang M P, Sahin A A, Ordonez N G, Sneige N. HER-2/neu gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast cancer. *Am J Clin Pathol* 2000;113:852-859.

Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. *Cancer Res* 1995;55:3068-3072.

Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Androgen receptor status of lymph node metastases from prostate cancer. *Prostate* 1996;28:129-135.

Hobisch A, Eder I E, Putz T, Horninger W, Bartsch G, Klocker H, Culig Z. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res* 1998;58:4640-4645.

Huang Q, Schanta S P, Rao P H, Mo J, McCormack S A, Chaganti R S K. Improved degenerate oligonucleotide primed PCR-comparative genomic hybridization for analysis of DNA copy number changes in tumors. *Genes Chromosomes Cancer* 2000;28:395-403.

Huggins C, Stevens R A. The effect of castration on benign hypertrophy of the prostate in man. *J Urol* 1940;43:705-714.

Huggins C, Hodges C V. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* 1941;1:293-297.

Hytytinen E, Visakorpi T, Kallioniemi A, Kallioniemi O P, Isola J. Improved technique for analysis of formalin-fixed paraffin-embedded tumours by fluorescence in situ hybridization. *Cytometry* 1994;16:93-99.

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. cDNA cloning of a novel androgen receptor subtype. *J Biol Chem* 1999;274:25205-25209.

Ikonen T, Palvimo J J, Kallio P J, Reinikainen P, Janne O A. Stimulation of androgen-regulated transactivation by modulators of protein phosphorylation. *Endocrinology* 1994;135:1359-1366.

Imperato-McGinley J, Gautier T, Zinisky T. Prostate visualization studies in males homozygous or heterozygous for 5 alpha-reductase deficiency. *J Clin Endocrinol Metab* 1992;75:1022-1026.

Ingle J N, Tormey D C, Tan H K. The bone marrow examination in breast cancer; diagnostic considerations and clinical usefulness. *Cancer* 1978;41:670-674.

Irvine R A, Ma H, Yu M C, Ross R K, Stallcup M R, Coetzee G A. Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. *Hum Mol Genet* 2000;9:267-274.

Isola J, DeVries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. *Am J Pathol* 1994;145:1301-1308.

Jacob T W, Gown A M, Yaziji H, Barnes M J, Schnitt S J. Comparison of fluorescence *in situ* hybridization and immunochemistry for the evaluation of *HER-2/neu* in breast cancer. *J Clin Oncol* 1999;17:1974-1982.

James L, Varley J. Preparation, labelling and detection of DNA from archival sections suitable for comparative genomic hybridization. *Chromosome Res* 1996;4:163-164.

Janulis L, Grayhack J T, Lee C. Endocrinology of the Prostate. In: Prostatic Diseases. Lepor H (Ed), W. B. Saunders 2000.

Jenster G, van der Korput J A, van Vroonhoven C, van der Kwast T H, Trapman J, Brinkmann A O. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol Endocrinol* 1991;5:1396-1404.

Jenster G, Trapman J, Brinkmann A O. Nuclear import of the human androgen receptor. *Biochem J* 1993;293:761-768.

Jenster G, de Ruiter P E, van der Korput J A, Kuiper G G, Trapman J, Brinkmann A O. Changes in the abundance of androgen receptor isotypes: effects of ligand treatment, glutamine-stretch variation, and mutation of putative phosphorylation sites. *Biochemistry* 1994;33:14064-14072.

Jie-ping W, Fang-liu G. The prostate 41-65 years post castration, an analysis of 26 eunuchs. *Chinese Med J* 1987;100:271-272.

Joos S, Bergerheim U S, Pan Y, Matsuyama H, Bentz M, du Manoir S, Lichter P. Mapping of chromosomal gains and losses in prostate cancer by comparative genomic hybridization. *Genes Chromosomes Cancer* 1995;14:267-276.

Kallioniemi O P, Kallioniemi A, Sudar D, Rutowitz D, Gray J W, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumours. *Science* 1992;258:818-821.

Kallioniemi O-P, Kallioniemi A, Sudar D, Rutovitz D, Gray J W, Waldman F, Pinkel D. Comparative genomic hybridization: a rapid new method for detecting and mapping DNA amplification in tumours. *Semin Cancer Biol* 1993;4:41-46.

Kallioniemi O-P, Kallioniemi A, Piper J, Isola J, Waldman F M, Gray J W, Pinkel D. Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumours. *Genes Chromosom Cancer* 1994;10:231-243.

Kallioniemi O P, Visakorpi T. Genetic basis of clonal evolution of human prostate cancer. *Adv Cancer Res* 1996;68:225-255.

Kaltz-Wittmer C, Klenk U, A. G, Aust D E, Diebold J, Lohrs U, Baretton G B. FISH analysis of gene aberrations (MYC, CCND1, ERBB2, RB, and AR) in advanced prostate cancer before and after androgen deprivation therapy. *Lab Invest* 2000;80:1455-1464.

Kantoff P, Giovannucci E, Brown M. The androgen receptor CAG repeat polymorphism and its relationship to prostate cancer. *Biochim Biophys Acta* 1998;1378:C1-C5.

Karan D, Lin M F, Johansson S L, Batra S K. Current status of the molecular genetics of human prostatic adenocarcinomas. *Int J Cancer* 2003;103:285-293.

Kasahara K, Taguchi T, Yamasaki I, Kamada M, Yuri K, Shuin T. Detection of genetic alterations in advanced prostate cancer by comparative genomic hybridization. *Cancer Genet Cytogenet* 2002;137:59-63.

Kay E W, Walsh C J, Cassidy M, Leader M. C-erbB-2 immunostaining: problems with interpretation. *J Clin Pathol* 1994;47:816-822.

Kim S-H, Godfrey T, Jensen R H. Whole genome amplification and molecular genetic analysis of DNA from paraffin-embedded prostate adenocarcinoma tumor tissue. *J Urol* 1999;162:1512-8.

Kim S-H, Kim M S, Jensen R H. Genetic alterations in microdissected prostate cancer by comparative genomic hybridization. *Prostate Cancer Prostatic Dis* 2000;3:110-114.

Kimura N, Mizokami A, Oonuma T, Sasano H, Nagura H. Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J Histochem Cytochem* 1993;41:671-678.

King W, Proffitt J, Morrison L, Piper J, Lane D, Seelig S. The role of fluorescence in situ hybridization in molecular diagnosis and disease management. *Mol Diagn* 2000;5:309-319.

Kinsel L, Szabo E, Greene G L, Konrath J, Leight G S, McCarty Jr. K S. Immunocytochemical analysis of estrogen receptors as a predictor of prognosis in breast cancer patients: comparison with quantitative methods. *Cancer Res* 1989;49:1052-1056.

Klein C A, Schmidt-Kittler O, Schardt J A, Pantel K, Speicher M R, Riethmuller G. Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. *Proc Natl Acad Sci USA* 1999;96:4494-4499.

Klein C A, Seidl S, Petat-Dutter K, Offner S, Geigl J B, Schmidt-Kittler O, Wendler N, Passlick B, Huber R M, Schlimok G, Baeuerle P A, Riethmuller G. Combined transcriptome and genome analysis of single micrometastatic cells. *Nature Biotech* 2002;20:387-392.

Koivisto P, Hytyinen E, Palmberg C, Tammela T, Visakorpi T, Isola J, Kallioniemi O P. Analysis of genetic changes underlying local recurrence of prostate carcinoma during androgen deprivation therapy. *Am J Pathol* 1995;147:1608-1614.

Koivisto P A. Molecular cytogenetics of prostate cancer. *Crit Rev Oncogenesis* 1996;7:143-150.

Koivisto P, Kononen J, Palmberg C, Tammela T, Hytyinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi O P. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res* 1997;57:314-319.

Koivisto P, Kolmer M, Visakorpi T, Kallioniemi O P. Androgen receptor gene and hormonal therapy failure of prostate cancer. *Am J Pathol* 1998;152:1-9.

Koivisto P A, Helin H J. Androgen receptor gene amplification increases tissue PSA protein expression in hormone-refractory prostate carcinoma. *J Pathol* 1999;189:219-223.

Krithivas K, Yurgalevitch S M, Mohr B A, Wilcox C J, Batter S J, Brown M, Longcope C, McKinlay J B, Kantoff P W. Evidence that the CAG repeat in the androgen receptor gene is associated with the age-related decline in serum androgen levels in men. *J Endocrinol* 1999;162:137-142.

Kuduk S D, Harris T C, Zheng F F, Sepp-Lorenzino L, Ouerfelli Q, Rosen N, Danishefsky S J. Synthesis and evaluation of geldanamycin-testosterone hybrids. *Bioorg Med Chem Lett* 2000;10:1303-1306.

Kuiper G G, Faber P W, van Rooij H C, van der Korput J A, Ris-Stalpers C, Klaassen P, Trapman J, Brinkmann A O. Structural organization of the human androgen receptor gene. *J Mol Endocrinol* 1989;2:R1-R4.

Kuiper G G, de Ruiter P E, Grootegoed J A, Brinkmann A O. Synthesis and post-translational modification of the androgen receptor in LNCaP cells. *Mol Cell Endocrinol* 1991;80:65-73.

Kyprianou N, English H F, Isaacs J T. Programmed cell death during regression of PC-82 prostate cancer following androgen ablation. *Cancer Res* 1990;50:3748-3753.

Labrie F, Belanger A, Dupont A, Luu-The V, Simard J, Labrie C. Science behind total androgen blockade: from gene to combination therapy. *Clin Invest Med* 1993;16:475-492.

Langley E, Kemppainen J A, Wilson E M. Intermolecular NH₂-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. *J Biol Chem* 1998;273:92-101.

Lavinsky R M, Jepsen K, Heinzel T, Torchia J, Mullen T M, Schiff R, Del-Rio A L, Ricote M, Ngo S, Gemsch J, Hilsenbeck S G, Osborne C K, Glass C K, Rosenfeld M G, Rose D W. Diverse signalling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* 1998;95:2920-2925.

Lebeau A, Deimling D, Kaltz C, Sendelhofert A, Iff A, Luthardt B, Untch M, Lohrs U. HER-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. *J Clin Oncol* 2001;19:354-363.

Lee C. Role of androgen in prostate growth and regression: stromal-epithelial interaction. *Prostate (Suppl)* 1996;6:52-56.

Lee C. Biology of the prostatic ductal system. In: *Prostate: basic and clinical aspects*. Naz RK (Ed), CRC Press 1997.

Li H, Chen J D. The receptor-associated coactivator 3 activates transcription through CREB-binding protein recruitment and autoregulation. *J Biol Chem* 1998;273:5948-5954.

Linja M J, Savinainen K J, Saramaki O R, Tammela T, Vessella R L, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res* 2001;61:3550-3555.

Loda M, Fogt F, French F S, Posner M, Cukor B, Aretz H T, Alsaigh N. Androgen receptor immunohistochemistry on paraffin-embedded tissue. *Mod Pathol* 1994;7:388-391.

Logothetis C J, Vogelzang N J. Current treatment and investigative approaches to the management of hormone-refractory prostate cancer. In: *Genitourinary Oncology* (2nd Edition). Vogelzang N J, Scardino P T, Shilpey W U, Coffey D S (Ed). Philadelphia, Lippincott Williams and Wilkins 2000.

Lubahn D B, Joseph D R, Sullivan P M, Willard H F, French F S, Wilson E M. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* 1988;240:327-330.

Magi-Galluzzi C, Xu X, Hlatky L, Hahnfeldt P, Kalpan I, Hsiao P-W, Chang C, Loda M. Heterogeneity of androgen receptor content in advanced prostate cancer. *Mod Pathol* 1997;10:839-845.

Majeed A, Babb P, Jones J, Quinn M. Trends in prostate cancer incidence, mortality and survival in England and Wales 1971-1998. *Br J Urol Int* 2000;85:1-5.

Manning J E, Hershey N D, Brooker T R, Pellegrini M, Mitchell H K, Davidson N. A new method of *in situ* hybridization. *Chromosoma* 1975;53:107-117.

Mansi J L, Berger U, Wilson P, Shearer R, Coombes R C. Detection of tumour cells in bone marrow of patients with prostatic carcinoma by immunocytochemical techniques. *J Urol* 1988;139:545-548.

Masai M, Sumiya S, Akimoto S, Yatani R, Chang C, Liao S, Shimazaki J. Immunohistochemical study of androgen receptor in benign hyperplastic and cancerous human prostates. *Prostate* 1990;17:293-300.

Mattfeldt T, Wolter H, Kemmerling R, Gottfried H W, Kestler H A. Cluster analysis of comparative genomic hybridization (CGH) data using self-organizing maps: application to prostate carcinomas. *Anal Cell Pathol* 2001;23:29-37.

McCrea L E, Karaffin L. Carcinoma of the prostate: metastases, therapy and survival-a statistical analysis of 500 cases. *Int Coll Surg J* 1958;29:723-728.

McKenna N J, Lanz R B, O'Malley B W. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 1999;20:321-344.

McKenna N J, Xu J, Nawaz Z, Tsai S Y, Tsai M-J, O'Malley B W. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol* 1999;69:3-12.

McLeod D G, Crawford E D, DeAnton E P. Combined androgen blockade: the gold standard for metastatic prostate cancer. *Eur Urol* 1997;32(Suppl 3):70-77.

McPhaul M J, Marcelli M, Zoppi S, Griffin J E, Wilson J D. Genetic basis of endocrine disease. 4. The spectrum of mutations in the androgen receptor gene that causes androgen resistance. *J Clin Endocrinol Metab* 1993;76:17-23.

Mehan D J, Broun G O, Hoover B, Storey G. Bone marrow finding in carcinoma of the prostate. *J Urol* 1966;95:241-244.

Mettlin C. Clinical oncology update: prostate cancer. *Eur J Cancer* 1997;33:340-347.

Meyer M E, Gronemeyer H, Turcotte B, Bocquel M T, Tasset D, Chambon P. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* 1989;57:433-442.

Meyer W J, Migneon B R, Migneon C J. Locus on human X chromosome for dihydrotestosterone receptor and androgen insensitivity. *Proc Natl Acad Sci USA* 1975;72:1469-1472.

Miller K. Immunocytochemical techniques. In: Theory and practice of histological techniques (5th Edition). Bancroft JD, Gamble ME (Ed). London, Churchill Livingstone 2000.

Miyoshi Y, Uemura H, Fujinami K, Mikata K, Harada M, Kitamura H, Koizumi Y, Kubota Y. Fluorescence *in situ* hybridization evaluation of c-myc and androgen receptor gene amplification and chromosome anomalies in prostate cancer in Japanese patients. *Prostate* 2000;43:225-232.

Molina R, Ciocca D R, Tandon A T, Allred D C, Clark G M, Chamness G C, Gullick W J, McGuire W L. Expression of HER-2/neu oncoprotein in human breast cancer: a comparison of immunohistochemical and Western blot techniques. *Anticancer Res* 1992;12:1965-1972.

Molino A, Colombatti M, Bonetti F, Zardini M, Pasini F, Perini A, Pelosi G, Tridente G, Veneri D, Cetto G L. A comparative analysis of three different techniques for the detection of breast cancer cells in bone marrow. *Cancer* 1991;67:1033-1036.

Montie J E, Pienta K J. Review of the role of androgenic hormones in the epidemiology of benign prostatic hyperplasia and prostate cancer. *Urology* 1994;43:892-899.

Moore R A. Benign hypertrophy and carcinoma of the prostate. *Surgery* 1944;16:152-167.

Mostofi F K, Sesterhenn I A, Davis Jr. C J. Prostatic carcinoma: problems in the interpretation of prostatic biopsies. *Hum Pathol* 1992;23:223-241.

MRC Radiotherapy Working Group. A randomised trial of high dose therapy in localised carcinoma of the prostate using conformal radiotherapy techniques, MRC 2000.

Murphy W M, Soloway M S, Barrows G H. Pathologic changes associated with androgen deprivation therapy for prostate cancer. *Cancer* 1991;68:821-828.

Nam R K, Elhaji Y, Krahn M D, Hakimi J, Ho M, Chu W, Sweet J, Trachtenberg J, Jewett M A, Narod S A. Significance of the CAG repeat polymorphism of the androgen receptor gene in prostate cancer progression. *J Urol* 2000;164:567-572.

Nath J, Johnson K L. A review of fluorescence in situ hybridization (FISH): current status and future prospects. *Biotech Histochem* 2000;75:54-78.

Nazareth L V, Weigel N L. Activation of the human androgen receptor through a protein kinase A signalling pathway. *J Biol Chem* 1996;271:19900-19907.

Nazareth L V, Stenoien D L, Bingman 3rd W E, James A J, Wu C, Zhang Y, Edwards D P, Mancini M, Marcelli M, Lamb D J, Weigel N L. A C619Y mutation in the human androgen receptor causes inactivation and mislocalization of the receptor with concomitant sequestration of SRC-1 (steroid receptor coactivator 1). *Mol Endocrinol* 1999;13:2065-2075.

Nelson C M K, Boatman D L, Flocks R H. Bone marrow examination in carcinoma of the prostate. *J Urol* 1973;109:667-670.

Nelson K A, Witte J S. Androgen receptor CAG repeats and prostate cancer. *Am J Epidemiol* 2002;155:883-890.

Newmark J R, Hardy D O, Tonb D C, Carter B S, Epstein J I, Isaacs W B, Brown T R, Barrack E R. Androgen receptor gene mutations in human prostate cancer. *Proc Natl Acad Sci USA* 1992;89:6319-6323.

NHS Cancer Screening Program (2001). Guidelines for Non-operative Diagnostic Procedures and Reporting in Breast Cancer Screening. Sheffield, NHS Cancer Screening Programmes, NHSBSP Publication No 50.

Nieh P T. Withdrawal phenomenon with the antiandrogen Casodex. *J Urol* 1995;153:1070-1072.

Nowell P C. Mechanisms of tumour progression. *Cancer Res* 1986;46:2203-2207.

Nupponen N N, Kakkola L, Koivisto P, Visakorpi T. Genetic alterations in hormone-refractory recurrent prostate carcinomas. *Am J Pathol* 1998;153:141-8.

Nupponen N N, Visakorpi T. Molecular biology of progression of prostate cancer. *Eur Urol* 1999;35:351-354.

Nupponen N N, Visakorpi T. Molecular cytogenetics of prostate cancer. *Microsc Res Tech* 2000;51:456-463.

Oñate S A, Tsai S Y, Tsai M-J, O'Malley B W. Sequence and characterization of a co-activator for the steroid hormone receptor superfamily. *Science* 1995;270:1354-1357.

Palmberg C, Koivisto P, Kakkola L, Tammela T L J, Kallioniemi O P, Visakorpi T. Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. *J Urol* 2000;164:1992-1995.

Perez E A, Roche P C, Jenkins R B, Reynolds C A, Halling C A, Ingle J N, Wold L E. HER2 testing in patients with breast cancer: poor correlation between weak positivity by immunohistochemistry and gene amplification by fluorescence in situ hybridization. *Mayo Clin Proc* 2002;77:148-154.

Pertschuk L P, Macchia R J, Feldman J G, Brady K A, Levine M, Kim D S, Eisenberg K B, Rainford E, Prins G S, Greene G L. Immunocytochemical assay for androgen receptors in prostate cancer: a prospective study of 63 cases with long-term follow-up. *Ann Surg Oncol* 1994;1:495-503.

Pertschuk L P, Schaeffer H, Feldman J G, Macchia R J, Kim Y D, Eisenberg K, Braithwaite L V, Axiotis C A, Prins G, Greene G L. Immunostaining for prostate cancer androgen receptor in paraffin identifies a subset of men with a poor prognosis. *Lab Invest* 1995;73:302-305.

Piper J, Rutovitz D, Sudar D, Kallioniemi A, Kallioniemi O-P, Waldman F M, Gray J W, Pinkel D. Computer image analysis of comparative genomic hybridization. *Cytometry* 1995;19:10-26.

Porkka K, Saramaki O, Tanner M, Visakorpi T. Amplification and overexpression of Elongin C gene discovered in prostate cancer by cDNA microarrays. *Lab Invest* 2002; 82:629-637.

Porter A T, McEwan A J, Powe J E, Reid R, McGowan D G, Lukka H, Sathyaranayana J R, Yakemchuk V N, Thomas G M, Erlich L E. Results of a randomised phase III trial to evaluate the efficacy of strontium-89 adjuvant to local field external beam irradiation in the management of endocrine resistant metastatic prostate cancer. *Int J Radiat Oncol Biol Phys* 1993;25:805-813.

Pratt W B. The role of heat shock proteins in regulating the function, folding and trafficking of the glucocorticoid receptor. *J Biol Chem* 1993;268:21455-21458.

Press M F, Hung G, Godolphin W, Slamon D J. Potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res* 1994;54:2771-2777.

Prins G S, Jung M H, Vellanoweth R L, Chatterjee B, Roy A K. Age-dependant expression of the androgen receptor gene in the prostate and its implications in glandular differentiation and hyperplasia. *Dev Genetics* 1996;18:99-106.

Prins G S. Molecular biology of the androgen receptor. *Mayo Clin Proc* 2000;75(Suppl):S32-S35.

Prostate Cancer Trialists' Collaborative Group. Maximum androgen blockade in advanced prostate cancer: an overview of the randomised trials. *Lancet* 2000;355:1491-1498.

Provan A B, Hodges E, Smith A G. Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. *J Clin Pathol* 1992;45:763-765.

Qian J, Bostwick D G, Takahashi S, Borell T J, Herath J F, Lieber M M, Jenkins R B. Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence *in situ* hybridization. *Cancer Res* 1995;55:5408-5414.

Qian J, Bostwick D G, Takahashi S, Borell T J, Brown J A, Lieber M M, Jenkins R B. Comparison of fluorescence *in situ* hybridization analysis of isolated nuclei and routine histological sections from paraffin-embedded prostatic adenocarcinoma specimens. *Am J Pathol* 1996;149:1193-1199.

Raffo A J, Perlman H, Chen M W, Day M L, Streitman J S, Buttyan R. Overexpression of bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo*. *Cancer Res* 1995;55:4438-4445.

Reese D M, Slamon D J. HER-2/neu signal transduction in human breast and ovarian cancer. *Stem Cells* 1997;15:1-8.

Reiner A, Neumeister B, Spona J, Reiner G, Schemper M, Jakesz R. Immunocytochemical localization of estrogen and progesterone receptor and prognosis in human primary breast cancer. *Cancer Res* 1990;50:7057-7061.

Rohr K, Hegglin R. Tumorzellen im sternalpunktat. *Deutsch Arch f klin Med* 1936;179:61-79.

Rokman A, Koivisto P A, Matikainen M P, Kuukasjarvi T, Poutiainen M, Helin H J, Karhu R, Kallioniemi O P, Schleutker J. Genetic changes in familial prostate cancer by comparative genomic hybridization. *Prostate*. 2000;146:233-239.

Ruizeveld de Winter J A, Trapman J, Vermey M, Mulder E, Zegers N D, van der Kwast T H. Androgen receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem* 1991;39:927-936.

Ruizeveld de Winter J A, Janssen P J, Sleddens H M, Verleun-Mooijman M C, Trapman J, Brinkmann A O, Santerse A B, Schroder F H, van der Kwast T H. Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. *Am J Pathol* 1994;144:735-746.

Russell P J, Bennett S, Strickler P. Growth factor involvement in the progression of prostate cancer. *Clin Chem* 1998;44:705-23.

Sabbatini P, Larson S M, Kremer A, Zhang Z F, Sun M, Yeung H, Embriaco M, Horak I, Conolly M, Ding C, Ouyang P, Kelly W K, Scher H I. Prognostic significance of extent of disease in bone in patients with androgen-independent prostate cancer. *J Clin Oncol* 1999;17:948-957.

Sadar M D. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. *J Biol Chem* 1999;274:7777-7783.

Sadi M V, Walsh P C, Barrack E R. Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy. *Cancer* 1991;67:3057-3064.

Sadi M V, Barrack E R. Image analysis of androgen receptor immunostaining in metastatic prostate cancer. Heterogeneity as a predictor of response to hormonal therapy. *Cancer* 1993;71:2574-2580.

Saramaki O, Willi N, Bratt O, Gasser T C, Koivisto P, Nupponen N N, Bubendorf L, Visakorpi T. Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. *Am J Pathol* 2001;159:2089-2094.

Saramaki O R, Savinainen K J, Nupponen N N, Bratt O, Visakorpi T. Amplification of hypoxia-inducible factor 1alpha gene in prostate cancer. *Cancer Genet Cytogenet* 2001;128:31-34.

Sarsfield P, Wickham C L, Joyner M V, Ellard S, Jones D B, Wilkins B S. Formic acid decalcification of bone marrow trephines degrades DNA: alternative use of EDTA allows the amplification and sequencing of relatively long PCR products. *Mol Pathol* 2000;53:336.

Sattler H P, Rohde V, Bonkhoff H, Zwergel T, Wullich B. Comparative genomic hybridization reveals DNA copy number gains to frequently occur in human prostate cancer. *Prostate* 1999;39:79-86.

Sattler H P, Lensch R, Rohde V, Zimmer E, Meese E, Bonkhoff H, Retz M, Zwergel T, Bex A, Stoeckle M, Wullich B. Novel amplification unit at chromosome 3q25-q27 in human prostate cancer. *Prostate*. 2001;45:207-215.

Sausville E A. Gene amplification in small cell carcinoma of the lung. In: *Gene amplification in mammalian cells. a comprehensive guide* Kellens RE (Ed). New York, Marcel Dekker, Inc. 1993.

Savinainen K J, Saramaki O R, Linja M J, Bratt O, Tammela T L, Isola J J, Visakorpi T. Expression and gene copy number analysis of ERBB2 oncogene in prostate cancer. *Am J Pathol* 2002;160:339-345.

Scher H I, Kelly W K. Flutamide withdrawal syndrome: its impact on clinical trials in hormone-refractory prostate cancer. *J Clin Oncol* 1993;15:66-1572.

Schirrmeister H, Guhlmann A, Elsner K, Kotzerke J, Glatting G, Rentschler M, Neumaier B, Trager H, Nussle K, Reske S N. Sensitivity in detecting osseous lesions depends on anatomical localization: planar bone scintigraphy versus 18F PET. *J Nucl Med* 1999;40:1623-1629.

Schnitt S J, Jacob T W. Current status of HER2 testing. Caught between a rock and a hard place. *Am J Clin Pathol* 2001;116:806-810.

Scott W W. What makes the prostate grow? *J Urol* 1953;70:477-488.

Shannon J M, Cunha G R. Autoradiographic localization of androgen receptors in the developing mouse prostate. *Prostate* 1993;4:367-373.

Shemshedini L, Knauthe R, Sassone-Corsi P, Pernon A, Gronemeyer H. Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. *EMBO J* 1991;3839-3849.

Shibata H, Spencer T E, Oñate S A, Jenster G, Tsai T, Tsai M-J, O'Malley B W. Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog Horm Res* 1997;52:141-165.

Siiteri P K, Wilson J D. Testosterone formation and metabolism during male sexual differentiation in the human embryo. *J Clin Endocrinol Metab* 1974;38:113-125.

Simental J A, Sar M, Lane M V, French F S, Wilson E M. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J Biol Chem* 1991;266:510-518.

Sinowatz F, Amselgrubner W, Plendl J, Kölle S, Neumüller C H, Boos G. Effects of hormones on the prostate in adult and aging men and animals. *Microsc Res Tech* 1995;30:282-292.

Slamon D J, Godolphin W, Jones L A, Holt J A, Wong S G, Keith D E, Levin W, Stuart S G, Udoce J, Ullrich A, Press M F. Studies of HER-2/neu proto-oncogene in human breast and ovarian cancers. *Science* 1989;244:707-712.

Smith D F, Toft D O. Steroid receptors and their associated proteins. *Mol Endocrinol* 1993;7:4-11.

Solit D B, Zheng F F, Drobnjak M, Munster P N, Higgins B, Verbel D, Heller G, Tong W, Cordon-Cardo C, Agus D B, Scher H I, Rosen N. 17-Allyamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin Cancer Res* 2002;8:986-993.

Soloway M S, Hardeman S W, Hickey D, Raymond J, Todd B, Soloway S, Moinuddin M. Stratification of patients with metastatic prostate cancer based on extent of disease on initial bone scan. *Cancer* 1988;61:195-202.

Speicher M R, du Manoir S, Schröck E, Holtgreve-Grez H, Schoell B, Lengauer C, Cremer T, Ried T. Molecular cytogenetic analysis of formalin-fixed, paraffin embedded solid tumours by comparative genomic hybridization after universal DNA-amplification. *Hum Mol Genet* 1993;2:1907-1914.

Spiers A S D, Deal D R, Kasimis B S, Miller B R. Evaluation of the bones and bone marrow in patients with metastatic carcinoma of the prostate: radiological cytologic and cytogenetic findings. *J Med* 1982;13:303-307.

Spiers V, Kerin M J. Prognostic significance of oestrogen receptor beta in breast cancer. *Br J Surg* 2000;87:405-409.

Srinivasan G, Campbell E, Bashirelahi N. Androgen, estrogen and progesterone receptors in normal and aging prostates. *Microsc Res Tech* 1995;30:293-304.

Stanford J L, Just J J, Gibbs M, Wicklund K G, Neal C L, Blumenstein B A, Ostrander E A. Polymorphic repeats in the androgen receptor gene: molecular markers of prostate cancer risk. *Cancer Res* 1997;57:1194-1198.

Steinborn M M, Heuck A F, Tiling R, Bruegel M, Gauger L, Reiser M F. Whole body bone marrow MRI in patients with metastatic disease to the skeletal system. *J Comput Assist Tomogr* 1999;23:123-129.

Steiner T, Junker K, Burkhardt F, Braunsdorf A, Janitzky V, Schubert J. Gain in chromosome 8q correlates with early progression in hormonal treated prostate cancer. *Eur Urol* 2002;41:167-171.

Suzuki H, Akakura K, Komiya A, Aida S, Akimoto S, Shimazaki J. Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome. *Prostate* 1996;29:153-158.

Sy F A, Gursel E O, Veenema R J. Positive random iliac bone biopsy in advanced prostate cancer. *Urology* 1973;11:125-127.

Takeda H, Akakura K, Masai M, Akimoto S, Yatani R, Shimazaki J. Androgen receptor content of prostate carcinoma cells estimated by immunohistochemistry is related to prognosis of patients with stage D2 prostate carcinoma. *Cancer* 1996;77:934-940.

Tanner M, Gancberg D, Di Leo A, Larsimont D, Rouas G, Piccart M J, Isola J. Chromogenic *in situ* hybridization: a practical alternative for fluorescence *in situ* hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. *Am J Pathol* 2000;157:1467-1472.

Taplin M E, Bubley G J, Shuster T D, Frantz M E, Spooner A E, Ogata G K, Keer H N, Balk S P. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N Engl J Med* 1995;332:1393-1398.

Taplin M E, Bubley G J, Ko Y J, Small E J, Upton M, Rajeshkumar B, Balk S P. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res* 1999;59:2511-2515.

Taplin M E, Shuk-Mei H. The endocrinology of prostate cancer. *J Clin Endocrinol Metab* 2001;86:3467-3477.

Thomson T A, Hayes M M, Spinelli J J, Hillard E, Sawrenko C, Phillips D, Dupuis B, Parker R L. HER2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent *in situ* hybridization. *Mod Pathol* 2001;14:1079-1086.

Tilley W D, Marcelli M, Wilson J D, McPhaul M J. Characterization and expression of a cDNA encoding the human androgen receptor. *Proc Natl Acad Sci USA* 1989;86:327-331.

Torchia J, Glass C, Rosenfeld M G. Co-activators and co-repressors in the integration of transcriptional responses. *Curr Opin Cell Biol* 1998;10:373-383.

Trapman J, Klaassen P, Kuiper G G, van der Korput J A, Faber P W, van Rooij H C, Geurts van Kessel A, Voorhorst M M, Mulder E, Brinkmann A O. Cloning, structure and expression of a cDNA encoding the human androgen receptor. *Biochem Biophys Res Commun* 1988;153:241-248.

Truss M, Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocr Rev* 1993;14:459-479.

Tsuda H. Prognostic and predictive value of cerbB-2 (HER-2/neu) gene amplification in human breast cancer. *Breast Cancer* 2001;8:38-44.

Tsuda H, Akiyama F, Terasaki H, Hasegawa T, Kurosumi M, Shimadzu M, Yanmamori S, Sakamoto G. Detection of HER-2/neu (c-erb B-2) DNA amplification in primary breast carcinoma. *Cancer* 2001;92:2965-2974.

van der Kwast T H, Schalken J, Ruizeveld de Winter J A, van Vroonhoven C C, Mulder E, Boersma W, Trapman J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int J Cancer* 1991;48:189-193.

van Doorn E, Craven S, Bruchovsky N. The relationship between androgen receptors and the hormonally controlled responses of rat ventral prostate. *Biochem J* 1976;160:11-21.

Vanaja D K, Mitchell S H, Toft D O, Young C Y. Effects of geldanamycin on androgen receptor function and stability. *Cell Stress Chaperones* 2002;7:55-64.

Varenhorst E, Alund G, Lindstrom E, Manson J C. Bone marrow aspiration biopsy and bone scanning in the staging of prostate cancer. *Br J Urol* 1983;55:534-537.

Veldscholte J, Ris-Stalpers C, Kuiper G G, Jenster G, Berrevoets C, Claassen E, van Rooij H C, Trapman J, Brinkmann A O, Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* 1990;173:534-540.

Verdorfer I, Hobisch A, Culig Z, Hittmair A, Bartsch G, Erdel M, Duba H C, Utermann G. Combined study of prostatic carcinoma by classical cytogenetic analysis and comparative genomic hybridization. *Int J Oncol* 2001;19:1263-1270.

Verhagen P C S M, Zhu X L, Rohr L R, Cannon-Albright L A, Tavtigian S V, Skolnick, M H, Brothman A R. Microdissection, DOP-PCR, and comparative genomic hybridization of paraffin-embedded familial prostate cancer. *Cancer Genet Cytogenet* 2000;122:43-48.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi O P. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet* 1995;9:401-406.

Visakorpi T, Kallioniemi A H, Syvanen A C, Hytyinen E-R, Karhu R, Tammela T, Isola J J, Kallioniemi O-P. Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. *Cancer Research* 1995;55:342-347.

Voeller H J, Wilding G, Gelmann E P. v-rasH expression confers hormone-independent growth in vitro to LNCaP prostate carcinoma cells. *Mol Endocrinol* 1991;5:209-216.

Vogelstein B, Kinzler K W. The multistep nature of cancer. *Trends Genet* 1993;9:138-141.

Wang S, Saboorian H, Frenkel E, Haley B, Siddiqui M T, Gokaslan S, Wians Jr. F H, Hynan L, Ashfaq R. Assessment of HER-2/neu status in breast cancer. *Am J Clin Pathol* 2001;116:495-503.

Ware M A, Abel P D, Madaan S, Stamp G W H, Lalani E. Evidence for amplification of the androgen receptor gene in prostate cancer prior to androgen ablation therapy. *Proc AACR* 2000;41:182.

Watters A D, Bartlett J M S. Quantitation of FISH signals in archival tissue. In: *Molecular Medicine, Volume 39: Ovarian Cancer: Methods and Protocols*. Bartlett J M S (Ed). Totowa, NJ, Humana Press 1999.

Welch J F, Mackinnery C C. Experience with aspiration biopsies of the bone marrow in the diagnosis and prognosis of carcinoma of the prostate gland. *Am J Clin Pathol* 1964;41:509-512.

Westlin P, Stattin P, Damberg J-E, Bergh A. Castration therapy induces apoptosis in a minority and decreases cell proliferation in a majority of human prostatic tumours. *Am J Pathol* 1995;146:1368-1375.

White J W. The results of double castration in hypertrophy of the prostate. *Ann Surg* 1895;22:1-80.

Wickham C L, Boyce M, Joyner M V, Sarsfield P, Wilkins B S, Jones D B, Ellard S. Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *Mol Pathol* 2000;53:9-23.

Wilson J D. The pathogenesis of benign prostatic hyperplasia. *Am J Med* 1980;68:745-756.

Wilson J D, Griffin J E, George F W, Leshin M. The role of gonadal steroids in sexual differentiation. *Recent Prog Horm Res* 1981;37:1-39.

Wilson J D, Griffin J E, Russell D W. Steroid 5-alpha-reductase 2 deficiency. *Endocr Rev* 1993;14:577-593.

Wilson J D. Role of dihydrotestosterone in androgen action. *Prostate (Suppl)* 1996;6:88-92.

Wolter H, Trijic D, Gottfried H W Mattfeldt T. Chromosomal changes in incidental prostatic carcinomas detected by comparative genomic hybridization. *Eur Urol* 2002;41:328-334.

Wolter H, Gottfried H W, Mattfeldt T. Genetic changes in stage pT2N0 prostate cancer studied by comparative genomic hybridization. *Br J Urol Int* 2002;89:310-316.

Wood Jr. D P, Banks E R, Humphreys S, McRoberts J W, Rangnekar V M. Identification of bone marrow micrometastases in patients with prostate cancer. *Cancer* 1994;74:2533-2540.

Yeh S, Lin H K, Kang H Y, Thin T H, Lin M F, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci USA* 1999;96:5458-5463.

Zhu X, Liu J P. Steroid-independent activation of androgen receptor in androgen-independent prostate cancer: a possible role for the MAP kinase signal transduction pathway? *Mol Cell Endocrinol* 1997;134:9-14.

Zitzelsberger H, Kulka U, Lehmann L, Walch A, Smida J, Aubele M, Lörch T, Höfler H, Bauchinger M, Werner M. Genetic heterogeneity in a prostate carcinoma and associated prostatic epithelial neoplasia as demonstrated by combined use of laser capture microdissection, degenerate oligonucleotide primed PCR and comparative genomic hybridization. *Virchows Arch* 1998;433:297-304.

Zitzelsberger H, Engert D, Walch A, Kulka U, Aubele M, Hofler H, Bauchinger M, Werner M. Chromosomal changes during development and progression of prostate adenocarcinomas. *Br J Cancer* 2001;84:202-208.

Zuckerman S. The endocrine control of the prostate. *Proc R Soc Med* 1936;29:1557-1567.

Appendix 1.

PATIENT INFORMATION SHEET UCL HOSPITALS TRUST



ANDROGEN (MALE HORMONE) RECEPTORS IN PROSTATE CANCER

We would like to invite you to take part in a study that is likely to increase our knowledge of prostate cancer.

Some time ago you were found to have a tumour of the prostate gland, which had shown signs of involving some of your bones. We know that this disease is sensitive to treatment with hormones and you will already have had a treatment that lowers the amount of circulating male sex hormone. Unfortunately your disease is no longer responding to this measure and we would now like to treat you with other hormones.

Exactly why a treatment stops working is unclear. Recently changes in the hormone receptor by which hormones become attached to tumour cells have been described and we would like to find out if this is the reason. These changes may also tell us which treatments will work best in the future.

We would like to analyse some of your tumour cells in the laboratory so we can study their hormone receptors. The best way for us to do this is for us to take a sample of your bone marrow from the top of your hipbone. This procedure is frequently performed. We will give you a local anaesthetic injection in your back in the region of your hipbone to minimise any pain, and we would then push a needle into the bone and withdraw a little marrow sample (it looks like blood) into a syringe. The procedure is not distressing, and the main complication is that there may be discomfort at the time the needle enters the bone and at the time when the marrow is withdrawn. There will be mild tenderness in the area for the next 24 hours. There is a small risk (less than 5 %) of a haematoma (bruising) and minimal risk of bleeding or infection.

This is not part of routine treatment and will not be of immediate benefit to you. You do not have to take part in this study if you do not want to. If you decide to take part you may withdraw at any time without having to give a reason. Your decision whether or not to take part or not will not affect your care and management in any way. An ethical committee reviews all proposals for research using patients before they can proceed. The joint UCL/UCLH Committee on the Ethics of Human Research reviewed this proposal.

If you would be willing to consider helping us with the study or have any questions please contact either Dr Harland on 020 7380 9041 or Dr Richard Brown on 020 7679 9296.



Institute of Urology Research Laboratory
3rd Floor
Charles Bell House
67 Riding House Street
London
W1N 7PN.

Consultant :

Consent Form for Bone Marrow Biopsy in Advanced Prostate Cancer Study.

Hospital Sticky Label:

I _____ have read the study information leaflet and had an opportunity to ask any questions that I may have about the bone marrow biopsy from the doctor listed below. I understand that it is a research procedure and agree to the procedure being done under a local anaesthetic.

Signed: _____
Patient Name.

Name: _____

Date: _____

Signed: _____
Doctor Name.

Name: _____

Date: _____



THE UNIVERSITY COLLEGE LONDON HOSPITALS NHS TRUST

CONFIDENTIAL

CONSENT TO A POST MORTEM EXAMINATION

I understand that -

- organs, parts of organs and tissues can be taken from the body in a post-mortem examination.
- tissue samples or fluids taken from the body may be held for laboratory examination.
- some samples for microscopic examination may be stored long-term for further study and checking

Section 1

CONSENT TO POST MORTEM

1. I give my consent to a post-mortem examination being carried out on the body of: in order to find the cause of death and study the effects of treatment.
2. Has the deceased or any other relative ever expressed an objection to a post-mortem examination, as far as you know?

Yes No

3. Do you wish to limit the examination to specific parts of the body?

Yes No

If Yes, please complete Section 6 of this form.

4. Do you wish to limit the organs being taken for further investigation?

Yes No

If Yes, please complete Section 7 of this form.

Section 2
PHOTOGRAPHY

5. Do you consent for photographs being taken of parts of the body?

Yes No

6. Do you consent to such photographs being displayed for medical research and educative purposes?

Yes No

Section 3

FETAL AND PERINATAL POST-MORTEM (including external assessment with the examination of the placenta)

7. Do you consent to a skin biopsy (sample) being performed which will then be used to examine cells?

Yes No

8. Do you consent to cells obtained by the above means being used in medical research?

Yes No

9. Do you consent for photographs being taken of the whole body (fetuses and babies only)?

Yes No

Section 4

MEDICAL RESEARCH AND EDUCATION

We may wish to retain and use some bodily material (organs, parts of organs and tissues) for an unlimited time for medical research and education. Please note that any such research will be approved by the Trust Ethics Committee before it is undertaken.

10. Do you consent to any bodily material (remaining tissues, organs, parts of organs or fluid) being taken and used for medical education and research?

Yes No

11. Do you consent to stored tissue blocks following diagnostic examination being taken and used for medical education and research?

Yes No

12. Do you consent to such bodily material (organs, parts of organs and tissues) being gifted to the hospital and displayed for research and educative purposes in the future?

Yes No

Section 5

DISPOSAL OF BODILY MATERIAL

After completion of all investigations, we will need to arrange for the disposal of any remaining tissues, organs, parts of organs or fluid. Please let us know how you would like such disposal to be arranged.

13. I would like:

- the hospital to dispose of the bodily material or organs in a lawful and respectful way.
- to arrange for the bodily material or organs to be disposed of in a lawful way myself.
- the tissue samples or organs to be reunited with the body before it is released, even though this may delay the funeral.

Before you sign the consent form below, please ensure that you have completed all the relevant questions in sections 1, 2, 3, 4 & 5:

Signed Relationship to the deceased..... Date.....

Consent taken by.....

Signed Designation..... Date.....

Consent witnessed by

Signed Designation..... Date.....

Designated Hospital contact..... Telephone number

**If you answered Yes to question 3 you will also need to complete and sign section 6.
If you answered Yes to question 4 you will also need to complete and sign section 7.**

Section 6

Only complete this section of the form if you answered “Yes” to question 3

LIMITED POST-MORTEM EXAMINATION

If you choose to limit the examination you should be aware that it may not be possible to ascertain the cause of death. The person who gave you this form will explain these options and their implications.

If 'Yes', to what part of the body do you wish the examination to be limited?

- Head
- Chest
- Abdomen
- Dissection without taking any material from the body (as defined above Section 1)

Section 7

Only complete this section of the form if you answered "Yes" to question 4

ORGANS BEING TAKEN AND HELD

I wish to limit consent to the following organs being taken (as noted below) for further investigation.

Brain Yes No

Heart Yes No

Other organs (please specify individually in the space provided below)

Signed Relationship to the deceased Date

Consent taken by _____

Signed Designation Date

Consent witnessed by _____

Signed Designation Date

Designated Hospital contact: _____ Telephone number: _____

Appendix 2. FISH CGH and Immunohistochemistry Protocols used in this Study.

1. Protocol for FISH on Formalin-Fixed, Paraffin-Embedded soft Tissue and Bone

Marrow Samples.

Pre-treatment methods for the two different sample types (bone and soft tissue) vary but the hybridisation steps are identical.

A. Pre-treatment for paraffin-embedded, formalin-fixed **Bone Marrow sections:**

1. Cut 5 μ m thickness sections and mount on silicon-coated slides.
2. Fill a Coplin jar with 0.2 M HCl (50 ml) and pre heat to 37°C in a water bath for use in step 13.
3. De-wax the slides in xylene twice for 10 minutes each time.
4. Rehydrate in methanol twice for 5 minutes each time.
5. While slides are in methanol, prepare antigen retrieval buffer (see recipe).
6. Put pressure cooker (Menerini Diagnostics, UK), without its lid, into the microwave containing 1L of antigen retrieval buffer (1L-distilled water, 0.37 g EDTA, 0.55 g Trisma Base, pH 8.0), and heat at full power for 15 minutes.
7. Place slides into water until the end of step 5.
8. Place the slides into the pressure cooker, making sure lid is properly sealed.
9. Microwave at full power until the pressure indicator rises (approximately 3 minutes), and then continue at full power for a further 5 minutes.

10. Remove pressure cooker and release the pressure (ensure you are wearing heat resistant gloves and visor).
11. Remove lid and allow to cool for 20 minutes.
12. Wash the slides in running cold tap water.
13. Add 25 mg of pepsin to 0.2 M HCl (0.05 % pepsin solution) in the water bath at 73°C.
14. Digest tissue in the 0.05% pepsin / 0.2 M HCl for 34 minutes at 37°C.
15. Wash the slides in distilled water.
16. Fix in 10 % neutral buffered formalin for 10 minutes at room temperature.
17. Rinse in water, then put through an alcohol series (70%, 85% and 100% IMS or ethanol).

18. Allow slides to air-dry whatever technique is used (manual or robot).

B. Pre-treatment for paraffin-embedded, formalin fixed **Soft Tissue sections:**

Prepare two water baths - one set at 37°C and one at 82 ° C for the pre-treatment.

1. Cut 5 µm thickness sections and mount on silicon-coated slides.
2. Dewax in xylene twice for 10 minutes each (this removes the wax from the processing steps).
3. Rehydrate in methanol twice for 5 minutes each (this step allows the sections to be introduced into aqueous solutions) and allow to air dry.

4. Treat with 0.2 M HCl at 82° C for 20 minutes.

This is a tissue permeabilisation agent (0.2M HCl is thought to increase the hybridisation signal as acid deproteinases the tissue, increasing probe penetration

This is due to the pH-dependent fixation reaction that proceeds more rapidly at higher pH, and is reversed following the introduction of an acid such as HCl.

Generally the use of a permeabilisation step reduces the proteolytic step allowing less tissue damage to occur.

5. Wash in 2 x SSC (pH 7) for 5 minutes.

6. Treat with 8% sodium thiocyanate at 80°C for 30 minutes (must be at correct temperature). Sodium thiocyanate is a strong reducing agent. Reducing agents break the protein disulphide bonds formed by formalin and allow greater nuclear accessibility.

7. Wash in 2 x SSC (pH 7) for 3 minutes.

8. Digest with 0.05 % pepsin at 30 °C for 34 minutes. Check temperature of solution first.

The optimum length of the digestion seems to vary slightly depending on the tissue used. It may require small empirical changes in the digestion time to give the best results.

Pepsin is primarily used as the proteolytic step in the demonstration of genomic DNA and some mRNA. Proteolytic digestion in combination with the 2 pre-treatment outlined above gives the optimal digestion.

9. Rinse in 2 x SSC (pH 7.0) 5 times.

10. Treat in 10% neutral buffered formalin for 10 minutes. It may seem like a contradiction to refix, but the short time in fixative appears to preserve the tissue following the pre-treatment and produces slightly better hybridisation results.
11. Wash in 2 x SSC (pH 7.0) for 5 minutes.
12. Dehydrate through an alcohol series (70%, (0%, 100%)) for 1 minute each.

If the Vysis VP2000 Robot is used for Pre-treatment of Slides for FISH, the following protocol is used. It replaces the manual steps 2-12:

The slides are taken through the following steps in an automated computerised robotic washer:

1. Xylene	5 minutes
2. Xylene	5 minutes
3. 95% Alcohol	5 minutes
4. 95% Alcohol (methanol)	5 minutes
5. 0.2N HCl	20 minutes
6. Water rinse	3 minutes
7. Vysis Pre-treatment solution (NaSCN pH 7.0, Vysis product number 30-801250)	30 minutes @ 80°C
8. Water rinse	5 minutes
9. Protease buffer (0.01 N HCl pH 2.0, Vysis product number 30-801255) & Protease 25 mg (0.05%)	26 minutes @37°C
10. Water rinse	3 minutes
11. Formalin fixation	10 minutes

12. Water rinse	3 minutes
13. 70% alcohol	5 minutes
14. 85% alcohol	5 minutes
15. 95% alcohol	5 minutes
16. Air Dry	

All robot washes are at room temperature unless otherwise stated.

Protease is kept at -20°C & added to the protease buffer 5 minutes before the slides enter the station.

All solutions are replaced after 5 washes.

13. Air dry at room temperature whatever method (manual or robot) is used.

C. Assessment of Tissue Digestion:

14. Examine the slides for degree of digestion: apply 0.25µg/ml DAPI in Vectashield antifade solution. If underdigested, redigest for 5 minutes; if overdigested re-do on a new sample with shorter digestion times.

15. Wash slides twice for 5 minutes each in distilled water and air dry (at 45°C)

D. Probe Hybridisation (for all types of specimen):

16. Make up the appropriate quantity of probe solution for the number of slides to be studied (one slide requires 10 µl of probe for a 22 x 22 mm coverslip or 20 µl for a 22 x 40 mm coverslip; 10 ml of probe equals 1 ml CepX chromosome probe, 1 ml AR probe, 1 ml water and 7 ml CEP buffer).
17. Apply probe to coverslip and place slide on top to spread probe onto the specimen.
18. Seal with rubber cement
19. Denature the slide and probe at 72°C for 5 minutes on a hot plate then hybridise overnight at 37°C in a humidified chamber (a Hybaid Omnislide slide hybridiser can do both of these steps as a program).

E. Post Hybridisation:

20. Peel off rubber cement and soak coverslip off in washing buffer (0.4X SSC, 0.3 % NP-40) at room temperature.
21. Incubate in a further wash using 0.4X SSC, 0.3 % NP-40 detergent for 2 minutes at 72°C.
22. Air dry slides.
23. Mount in 0.50 mg/ml DAPI in Vectashield antifade solution.
24. Seal with clear nail varnish.
25. Review under fluorescence microscope. Leica DM LB epifluorescence microscope with a Vysis Quad bandpass filter set (DAPI/Aqua/Green/Orange) (Part No 30-159080 for the Leica filter holder) and a Leica DC-200 camera and software.
26. Can be stored up to 6 months but best viewed and scored immediately.

2. Protocol for CGH on Formalin-Fixed, Paraffin-Embedded Soft Tissue and Bone Marrow Samples.

A full CGH experiment requires up to 14 days.

- A. Day 1&2: Sample & Slide preparation.
- B. Day 3: Laser Capture Microscopy (LCM) Protocol.
- C. Days 4-13: CGH Protocol.

A. Day 1&2: Sample and slide preparation.

Tissue sample and slide preparation should be done the day before each laser capture microscopy tissue extraction session, ensuring that samples are in optimum condition (i.e. no atmospheric absorbed moisture, reducing the effectiveness of the LCM).

Day 1: Slide cutting and mounting

1. From the formalin-fixed, paraffin-embedded tissue blocks previously collected (see Chapters 2 and 3), cut sections of 4-10 μm thickness in the standard way (4 μm gives better visual identification, 10 μm provides more DNA for extraction).
2. Mount each section on an uncoated slide without a cover slip.
3. Dry overnight at a low temperature 30 °C.

Day 2: Slide staining

1. Stain each slide with haematoxylin and eosin (H & E) in the standard way.
2. Briefly dehydrate each slide through an alcohol series (1 minute each 70%, 90%, 100%).
3. Place all slides in xylene for 5 minutes and allow to air dry.
4. Slides are now ready LCM.

B. Day 3: Laser Capture Microscopy.

Reagents:

- Gloves (to ensure your cells do not contaminate the sample)
- Recently prepared sample slides for tissue capture.
- LCM Microscope (Arturis PixCell II™).
- LCM CapSure® Caps, used to fuse the tissue samples onto the plastic caps containing a low melting point plastic on the inferior surface, to fuse and collect the tissue to be extracted.
- LCM CapSure® pads for cleaning the captured samples of unfused 'loose' tissue.
- Eppendorf tubes for every individual Cap tissue sample.
- Zip drive and disk for removal of photo documentation from the areas collected.

There are four basic components of a LCM:

1. A computer with a monitor to control the software.
2. The laser and associated electronics box.
3. The microscope system and camera attachment.
4. A separate viewing monitor.

Operating the LCM is divided into 5 sequential steps:

1. Entry into the system and setting up dissection parameters.
2. Microdissecting with the laser.
3. Harvesting the microdissected cells.
4. Saving the images (histology map photo, 'pre-capture' photo, 'post-capture' photo and 'cap' photo).
5. Shutting down the system.

A. Entry into the system and setting up dissection parameters.

Follow the start-up procedures as listed in the accompanying instructions for the LCM (UCL Rockefeller Building, 2nd Floor):

1. System entry password.
2. Starting the Arc2001 software.
3. System user selection to identify the individual user.
4. Setting up the 1st sample data folder.
5. Switching on the microscope, laser electronics and viewing monitor.

B. Microdissecting with the laser.

1. Place the tissue slide to be dissected, onto the microscope stage.
2. Ensure the area of the slide for dissection can be engaged by the stage vacuum system, holding the slide in place. Engage the vacuum and use the stage joystick to manoeuvre the slide for all subsequent movements.
3. Load a single CapSure® collection cap onto the capture transport arm from the cap cartridge. Place the diffuser lens over the cap and swing the transport capture arm over onto the microscope slide (necessary to ensure a good quality visual image).
4. Increase the microscope lighting to account for the diffuser lens, and focus using the x10 objective to produce a clear image on the viewing monitor.
5. Take a 'histology map' image of the specimen at x10 or x20 magnification.
6. Capture a further 'pre-capture' image at x60 magnification.
7. Identify the pink laser beam on the viewing monitor and adjust the size of the laser spot (7.5 μm , 15 μm or 30 μm diameter). Always focus the laser with a 7.5 μm spot size. A small tightly focused light beam with circular black borders should be seen.
8. Press the 'Enable laser' button on the laser control box.
9. Remove the diffuser lens from the transport arm and replace with the cap weight (necessary to ensure good apposition of the capture cap with the specimen).
10. Decrease the lighting on the microscope and refocus (the image will be correspondingly less sharp).

11. Using the viewing monitor to observe the sample fire the laser, simultaneously moving the stage joystick to make successive adjacent hits on the tissue sample (this requires some practice!).
12. Complete the capture by dissecting out an area in a logical fashion.

Evaluation of the Microdissection:

1. After completing the laser capture, lift up the capture transport arm, removing the cap from the slide. A small hole should be visible in the tissue at the site of the laser hits.
2. Remove the cap from the capture transport arm.
3. Place the diffuser lens back onto the capture transport arm and swing back over onto the tissue slide section.
4. Increase the microscope lighting again and after refocusing, take 'post-capture' photos at x20 and x 60 magnification.
5. Swing the capture transport arm away, remove the diffuser lens and switch off the vacuum system removing the slide with the residual tissue on it.
6. Replace with a clean blank microscope slide and re-engage the vacuum.
7. Re-insert the cap into the capture transport arm, place the diffuser lens over the cap and swing it back onto the blank slide.
8. Increase the microscope lighting again, refocus and take 'post-capture' photos at x20 and x 60 magnification.

C. Harvesting the Microdissected Cells.

1. Write the sample number on the completed CapSure® cap and onto a corresponding small Eppendorf Tube.
2. Position the cap over the Eppendorf tube, press down firmly, and rotate slightly.

The cap should be seated evenly on the tube and only inserted part-way (i.e. allowing sufficient security from contamination, but still allowing for the removal of the cap for addition of the tissue digestion mixture (see next section).

D. Saving the Images.

1. Transfer all images for each sample (histology map photo, 'pre-capture' photo, 'post-capture' photo and 'cap' photo) onto the Zip disk and remove the disk. Clear the images from the PC hard disk.

E. Closing down the system.

Reverse the process described in Section A to shut down the computer and equipment.

C. ***Days 4-14: CGH Protocol.***

Day 4: DNA Extraction.

Reagents:

- Proteinase K, 10 mg/ml.
- 1X Proteinase K extraction buffer (50mM KCl, 10mM Tris-HCl, pH8.0, 2.5mM MgCl₂, 0.1 mg/ml gelatin 0.45% NP-40, 0.45% Tween 20).
- Dilute proteinase K 1:20 in 1X proteinase K buffer. Make up immediately before use.

1. Extract DNA from the LCM caps by adding 20 μ l proteinase K in buffer extraction mixture into each Eppendorf with the cap as a lid.
2. Invert the LCM cap and Eppendorf tube so that the mixture sits on the cap.
3. Samples are held on ice until all aliquots of Proteinase K in extraction buffer are completed, ensuring even digestion times.
4. Digest overnight in a humidified chamber at 55°C.

Day 5: DNA Extraction, 1st DOP-PCR and checking the first PCR products.

(A, B, and C together require a full day)

Day 5A. Complete the DNA extraction:

1. The following morning, invert the tubes to right-way-up and vortex / centrifuge again.

2. Inactivate the extraction mixture by heating the tubes in a PCR machine at 95 °C for 10 minutes.
3. Centrifuge the samples again.
4. Store the extracted DNA at -20°C.

Day 5B. The First Degenerate Oligonucleotide Primer Polymerase Chain Reaction (DOP-PCR):

Reagents:

- Sterile Water
- 25mM Magnesium Chloride Solution (comes in the kit)
- 10 x PCR Buffer (comes in the kit)
- 2 mM Stock Nucleotide triphosphate (dNTPs) mix (adenosine-, cytosine-, guanine- and thymidine triphosphate).
- 100 µM Degenerate Oligonucleotide Primer (DOP)
- AmpliTaq Gold Taq 5 U / µl
- Mineral oil

1. For each separate tumour and normal sample of extracted DNA, add the quantities of reagents listed below into a separate small Eppendorf tube for each.
2. Make up a scaled up master mix of the reagents all together (e.g. x 8 if 7 samples).
3. Pipette 15 µl of the master mix into each Eppendorf tube.

NB. Work from top to bottom of the list adding the Taq last.

Reagent	$\times 1$ (25 μ l)	Final conc.
H_2O	10.0 μ l	
MgCl_2 (25 mM)	4.0 μ l	4.0 mM
10× PCR buffer	2.5 μ l	1×
dNTPs (2 mM each)	2.5 μ l	200 μ M ea.
DOP primer (100 μ M)	0.5 μ l	2.0 μ M
Taq (5 U/ μ l)	0.5 μ l	2.5 U
	20 μl	

* Plus 5 μ l DNA Sample = 25 μ l TOTAL VOLUME

5. Mix all the reagents (vortex and centrifuge).
6. Place a drop of mineral oil onto the mixture.
7. Place the final 5 μ l of DNA extraction sample carefully through the mineral oil layer into the aqueous layer.
8. Run in a PCR thermocycler machine set to the following program:
 - (94 °C, 9 minutes)
 - (94 °C 1 minute, 30 °C 1.5 minutes, 72 °C, 3 minutes) \times 8 cycles.
 - (94 °C 1 minutes, 62 °C 1 minute, 72 °C 1.5 minutes) \times 25 cycles.
 - (72 °C, 8 minutes)
 - Hold at 4 °C.

This PCR takes approximately 5 $^{1/2}$ hours to complete.

The DOP primer sequence is:

5'-CGACTCGAGNNNNNNATGTGG-3' (N =A,T,G or C in a random fashion). The N is important, as each different DOP (4^6 different DOPs in this case) will anneal with a large number of points along the genome helping to amplify all the genomic material up. The second aspect to this is the low (30 °C) PCR step, which allows a considerable degree of non-specific annealing to occur, further aiding amplification.

9. Store at DOP-PCR mixture at 4°C in the PCR till ready to run a gel to check the PCR product lengths to see if it is worth proceeding with the rest of the CGH.

Day 5C. Running on a Electrophoresis Gel to check PCR product lengths:

Reagents:

Agarose powder	Ethidium bromide
0.5 x TAE buffer	Type IV loading dye
1kB DNA Ladder	

1. Pipette out 5 μ l of each 25 μ l of PCR sample into separate small Eppendorf tubes.
2. Add 1.5 μ l of dye / buffer mix making a final volume of 6.5 μ l.
3. Vortex and centrifuge briefly.
4. For a Minigel (8 lanes, 6 samples, 1 bp ladder and 1 negative control), prepare an agarose gel.

5. Measure out 200 mls 0.5XTAE buffer.
6. Weight out 2 g of agarose to make up a 1 % gel.
7. Add the agarose to the TAE buffer and microwave for 4-5 minutes on full power until all agarose is dissolved.
8. Add 15 - 20 μ l of Ethidium bromide (EBr) to the agarose / TAE buffer and then allow the agarose solution to cool till able to touch the bottle by hand and then pour into a prepared gel mould.
9. Pour a gel of 6-7 mm thickness.
10. Allow to cool fully, removing the gel comb and tape that covers the side of the mould.
11. Place the gel into the electrophoresis tank (fully submerged). Make sure the buffer in the tank is 0.5X TAE as well. Place wells at the negative electrode end to run towards the positive electrode.
12. Add the DNA ladder to Well 1 with a micropipette (the ladder comes prelabelled with dye or mix 14 μ l type IV loading dye and 21 μ l ladder).
13. Into each subsequent well, add 6 μ l of the 6.5 μ l PCR sample.
14. Add into the final well the negative control (water, no DNA).
15. Run in the electrophoresis tank for 60 -80 minutes at 150 volts (check after 25-30 mins)
16. Remove the gel and expose to UV Light to expose the smear whose fragment lengths can be read from the ladder. Need > 1,000 bp, ideally 1,500 bp.> 2,000 bp is a luxury!
17. If suitable fragment size the samples can now be used for the second labelling DOP-PCR.

Day 6: Second (labelling) DOP-PCR & Probe preparation.

Day 6A. Second (labelling) DOP-PCR:

Reagents:

- Sterile Water
- 25mM Magnesium Chloride Solution (comes in the kit)
- 10 x PCR Buffer (comes in the kit)
- 2 mM A, C & G dNTPs, 0.5 mM dTTP
- 1 nmole/ μ l FluoroRed dTTP
- 1 nmole/ μ l FluoroGreen dTTP
- 100 μ M DOP
- AmpliTaq Gold Taq 5 U / μ l
- Mineral oil

1. For each separate tumour and normal sample of extracted DNA use a separate small Eppendorf tube and add the quantities of reagents listed below. Note that quantities are twice those of the 1st DOP-PCR.
2. In a similar way to the 1st DOP-PCR make up a scaled master mix of the reagents all together (e.g. x 8 if 7 samples, but one master mix will be needed for all normal samples (red fluorophore) and one for all tumour samples (green)).
3. Pipette 40 μ l of the master mix into each Eppendorf.

Add the fluorophores second to last (protection from light) and the Taq last.

	x1 (50 µl)	Final conc.
H₂O	17.0 µl	
MgCl₂ (25 mM)	8.0 µl	4.0 mM
10× PCR buffer	5.0 µl	1×
labelling dNTPs (2 mM each ACT, 0.5 mM dTTP)	5.0 µl	200 µM ea.
FluoroRed for Normal (1 nmole/µl)	3.0 µl	3 nmoles
FluoroGreen for Tumour (1 nmole/µl)	3.0 µl	3 nmoles
DOP primer (100 µM)	1.0 µl	2.0 µM
Taq (5 U/µl)	1.0 µl	5.0 U
	40 µl	

*** Plus 10 µl DNA Sample = 50 µl TOTAL VOLUME**

1. Once all the reagents are mixed (vortex and centrifuge) and 40 µl of master mix is placed into the Eppendorf tube, placed a drop of mineral oil onto the mixture.
2. Place a 10 µl of the sample from the 1st DOP-PCR sample carefully through the mineral layer into the aqueous layer to make up to a final volume of 50 µl for DOP-PCR.
3. Run in a PCR thermocycler machine set to the following program:
 - (94 °C, 4 minutes)
 - (94 °C 1 minute, 62 °C 1 minute, 72 °C 1.5 minutes) × 25 cycles
 - (72 °C 8 minutes)

- Hold at 4 °C.

This PCR takes approximately 2^{1/2} hours.

Day 6B: Probe preparation

Reagents:

- 100% ethanol
- 3M sodium acetate
- Cot-1 DNA (1 μ g/ μ l)
- Salmon sperm DNA (10 μ g/ μ l)

1. Once the second DOP-PCR has finished, precipitate the DNA probes (test and normal) over night at -20°C.
2. Precipitate the DNA into new Eppendorf tubes by adding:

100% ethanol	360 μ l
3M sodium acetate	16.4 μ l
Cot-1 DNA (1 μ g/ μ l)	60 μ l
Salmon sperm DNA (10 μ g/ μ l)	2 μ l
Test (tumour) DNA	50 μ l (i.e. all of it)
Control (normal) DNA	50 μ l (i.e. all of it)

Total Volume: 538.4 μ l.

3. You should see visible DNA strands appear in the mixture, which have a pink colouration (red fluorophore, the green can't be seen).

Cot-1 DNA binds near the centromere and stops red-green binding in this area, which degrades the image. Salmon sperm DNA is a carrier that helps the DNA to precipitate. As it doesn't contain any human DNA sequences it doesn't bind to Human DNA and the overall quantity is so small that it does not interfere with the reaction. Ethanol / Sodium acetate causes the precipitation to occur.

4. At this point place 100 mls 70% ethanol and a dry flat Coplin jar into a -20°C freezer for use the next day.

Day 7: Probe Hybridization Preparation.

(allow 3 $\frac{1}{2}$ hours)

Reagents:

- 70% Formamide/2XSSC Denaturing solution (350 μ l Formamide, 50 μ l 20XSSC, 100 μ l distilled water). Made fresh each time.
- Cow Gum or Halfords Rubber Cement

1. The following day, centrifuge the DNA suspension for 10 minutes at 13,500 rpm to produce a DNA pellet.
2. Dry thoroughly in air (but keep shielded from light).
3. Resuspended in 12 μ l hybridisation mixture. This step takes a long time to complete, as DNA is quite insoluble. Resuspend by placing the Eppendorf against the vibrating vortex mixer and flicking the tube with your finger. Allow a couple of minutes between attempts as this helps the resuspension process. If

still difficult to dissolve the probe can be placed in a thermocycler set at 55-60 °C to aid resuspension.

4. Once successfully resuspended the DNA probes are denatured by running again using the heating of a PCR machine at 75 °C for 15 minutes followed by 45 minutes at 37 °C.
5. Centrifuge briefly at the end of this step to collect any evaporate.
6. While this heating step is proceeding, take out a metaphase slide and allow to warm-up to room temperature.
7. Dehydrate in an alcohol series (70 %, 90% & 100 %), 5 minutes each.
8. Air dry.
9. Denature the metaphase slides in by using 100 µl of 70% formamide 2X SSC denaturing mixture under a 22 x 50 mm coverslip for 5 minutes at 75 °C using an accurate temperature controlled hot plate. This is best done by adding the 100µl onto the coverslip in a strip and placing the slide upside down over it. Apply gentle pressure to spread the denaturing solution then invert back up to right-way-up and place on the hot plate immediately. This denaturing step for the metaphase slide is the most critical step of the whole experiment and the 5 minutes time duration is critical (use a timer!). If some of the 100µl spills from under the coverslip move the slide to allow proper contact with the undersurface of the microscope slide with the hot plate.
10. Immediately remove the coverslip[s] (by tapping it gently onto a paper towel and place the slides directly into previously chilled 70% ethanol (-20 °C). Use a flat, rather than tall Coplin jar as it hold more ethanol, allowing more efficient cooling. Perform this step with the Coplin jar in immediate proximity to the slide to ensure the change is from 75°C to -20 °C as quickly as possible.

11. Place the -20 °C ethanol Coplin jar on a rocker for 5 minutes (timed accurately again).
12. Follow this by dehydration through an alcohol series (5 minutes each).
13. Allow to air dry.
14. Place 10 µl of probe added to the metaphase slides under a 22 mm diameter circular coverslip. Use the same technique for applying the coverslip as outlined above.
15. Once the cover slips are in place seal with cow gum (5 ml syringe and a cut down 200 µL pipette tip).
16. Incubated in a humidified lightproof chamber at 37 °C for **3 days**. Ensure humidification is generous by using a layer of paper towels well soaked in water in the box and place the slide on a plastic tip tray holder to keep it out of direct contact with the water.

Days 8 & 9: Probe Hybridization.

As listed immediately above.

Day 10: CGH Probe washes and DAPI counterstaining.

(allow 3 hours)

Reagents:

- 180 mls 50% formamide: 2X SSC (18 mls 20 x SSC, 72 mls distilled water & 90 mls formamide). Place 50-60 mls F/ SSC into a Coplin jar and the remaining solution into a bottle. Place both in the 45 °C water bath.
- 180 mls 2X SSC (18 mls 20 x SSC, 162 mls distilled water). Place 50-60 mls 2XSSC into a Coplin jar and place in the water bath. Put a further 50-60 mls 2XSSC into a Coplin jar and keep at room temperature. Put the remaining 50-60 mls into a bottle and place in the water bath.
- 60 mls TNT solution (6 mls 10XTN buffer, 54 mls distilled water and 150 µl Tween 20)

1. Remove the cow gum and the cover slip.
2. Wash the slides washed 3 times for 7, 10 & 13 minutes respectively in 50% formamide: 2X SSC at 45 °C. Discard the F/SSC after each wash. All water bath washes should be done with the water bath cover on to protect the fluorophore from light.
3. Wash in 2X SSC for 10 minutes twice at 45 °C and once at room temperature in a covered Coplin jar. Discard the 2XSSC after each wash. All the room temperature washes should also be done under a light proof conditions (Light proof covering jar) to protect the fluorophore from light.
4. Wash for 10 minutes in TNT solution on the rocker followed by 10 minutes in double distilled H₂O.
5. Dehydrate through alcohol series (70, 90 & 100 %, 5 minutes each) and allow to air dry.

6. Apply a counterstain (DAPI 0.2 mg/ml in Vectashield 4 μ l/ml) under a coverslip and store at 4 °C out of light for capture on an epifluorescence microscope as soon as possible.

Day 11: Fluorescence Microscopy & Metaphase Image Capture.

Reagents:

- Zeiss Axioskop fluorescence microscope.
- Photometrics KAF1400 Charged Coupled Device (CCD) camera.
- Mac Power PC computer with Vysis SmartCapture software.
- Quips® CGH Karyotyper / Analyser software (Applied Imaging).
- Zip or Jazz Drive and appropriate disk for transferring the captured metaphase images.

(All available at the Wolfson Institute laboratory, 2nd floor, Wolfson Institute, UCL)

1. Turn the analysis equipment on, ***in the following order*** (this must be done correctly), firstly the microscope UV lamp control box, secondly the filter wheel power box, and lastly the photometrics CCD camera control box. Then turn on the computer.
2. Open the “Quips FISH” or “IP Lab Spectrum” software.
3. Click on “Ext” from the top menu bar, and “capture” from the pull-down menu that appears.
4. Click on “flat field”. This produces a background flat field that is subtracted from the captured metaphase images for the final CGH analysis. Ensure that there are no

flat field images assigned to the Red, Green and Blue boxes by clicking on “none” in all of the drop down menus to the right of the respective colour boxes. Click “OK”.

5. In the “window name” box, type in “flat field” to denote new images for flat fielding.

6. Ensure all the boxes in the “normalisation” section are set to zero.

7. Check “keep originals” box. Make sure “interactive” is not checked.

8. For exposure times, set “black & white” to zero, and red, green and blue to any other number. Check all boxes as “auto”. “Gain” is set as 1 for black & white, and 4 for red, green and blue. Set “maximum exposure time” to 30 seconds.

9. Turn off all room lights, remove the slide from the lightproof storage box and place a slide for analysis on the stage.

10. Press “2” on the computer number pad to turn the filter wheel to blue (DAPI)^A

11. Locate a blank area on the slide, near a cell/metaphase^B.

12. Focus at $\times 100$ ^C on the cell/metaphase, then move to the blank area. *Ensure the area is blank under the green and red filters also.*

13. Pull out bar on microscope to switch the light from the binocular eyepieces to the camera.

14. Click the “expose” software button.

15. Click “flat field”. Assign “flat.R” to the “Red” box using the pull down menu to the right of the box. Similarly assign “flat.G” to “Green” and “flat.B” to “Blue”.

Click “OK”.

16. Uncheck the box “keep originals”.

17. Push in bar on microscope switching light from the camera back to the binocular eyepieces. Press “2” on the number pad to bring the filter wheel round to the blue filter again.

18. Find an appropriate high quality metaphase using $\times 20$ or $\times 60$ objective ^E
19. Focus at $\times 100$, checking that there is no artefactual fluorescence under the green (FITC filter) and red (rhodamine) filters.
20. Enter the slide name or identifying number and metaphase number in the software “window name” box.
21. Pull out bar on microscope switching light from the binocular lens to the camera. Click “expose”.
22. Click “done” when this has been completed.
23. Click on “File” from the top menu bar, and “save as” from the pull-down menu that appears.
24. Under “format”, pull down menu and click on “PICT” image format.
25. Rename the file, if necessary, and ensure the correct path to the Zip or Jaz drive to store the image. Click “save” ^f. Close all saved sample windows (*but not the flat field images*).
26. Click on “Ext” from the top menu bar, and “capture” from the pull-down menu that appears.
27. Repeat steps 17 to 26 until 5 –10 high quality metaphases have been saved for each experiment.
28. Click on “File” from the top menu bar, and “quit” from the pull-down menu that appears.
30. Shut down the computer, and turn off, *in the following order (this must be done correctly)*, firstly the photometrics CCD control box, secondly the filter wheel power box, and lastly the UV lamp control box. Record the total time the UV lamp has been on in the record book.

Notes:

^A Keypad number 3 turns the wheel to green (FITC filter) and keypad number 4 turns the filter wheel to red (rhodamine filter).

^B use $\times 20$ or $\times 60$ objective to scan the slide.

^C requires a drop of objective lens oil.

^D The criteria for selecting high quality metaphases include:

- DAPI staining that shows visible chromosome bands (consistent length of 400–550 bands with consistent chromosome condensation across the metaphase).
- Optimal chromosome spreading with few chromosomes that are bent, touching, or overlapping (some correcting for this can be done by the software prior to analysis).
- Balanced visible red and green fluorescence with a hybridisation pattern that is relatively smooth and non-granular, with adequate probe intensity and uniformity.
- Low binding of hybridized probes to chromosomal centromeres and heterochromatic regions.
- Minimal background cytoplasm surrounding the chromosomes.
- Background fluorescence that is low and uniform around each chromosome.

^E can be saved as native file format and converted later, if necessary.

Day12: Sample Processing with Vysis® CGH Software.

Reagents:

- Mac Power PC computer
- Quips® CGH Karyotyper / Analyser (Applied Imaging)
- Zip or Jazz Drive with appropriate disk containing the captured metaphase images.

1. Start the Macintosh computer and insert the Zip disk with the captured metaphases from the fluorescence microscope.
2. Start the IPLAB software.
3. Open the saved images and convert them to the IPS format required for the analysis.
4. Start the QUIPS CGH Karotyper software.
5. Sort the chromosomes images into pairs (1-22 and XY). The computer will attempt using automatic software recognition **but this must be checked visually yourself.**
6. Run the CGH software to produce the readout of gains and losses on the analysed chromosomes.

Ratio profiles are then compared to with visual assessment of the digital images to determine if they are concordant. Ratio changes that clearly exceed the background variation seen in normal controls are interpreted as evidence of real copy number differences. Telomeric, peri-centromeric or heterochromatic regions may fall outside the normal ± 1 S.D. range and are usually excluded from the analysis. Other ratio

changes at areas such as 1p32-pter, 16p, 17p, 19 and 22 need to be interpreted with caution because of high rates of apparently abnormal ratios in these regions in comparisons of two normal DNAs.

3. Protocol for Androgen Receptor Immunohistochemistry.

1. Cut 5 mm sections from routinely processed paraffin-embedded tissue.
2. Mounted on silicon-coated slides (Dako Product Number S2024)
3. Air dry at 60°C overnight.
4. Immerse the dried slides in distilled water.
5. Cook in 600mls of Dako Target Retrieval Solution, pH 9.0, (60 mls solution added to 540 mls distilled water, Dako Product Number S3307) in an 800W microwave at full power for 25 minutes (antigen retrieval step to unmask antigens).
6. Cool for 10 minutes, and then rinse in running tap water.
7. Place the slides into a slide cartridge of Dako TechMate® 500 processor and run on the following program (this capillary action, robotic slide process performs an automated sequence of 76 consecutive steps, to perform the immunohistochemistry, listed by the 3 or 4 digit/letter controlling computer codes):



Dako TechMate 500 Robotic Immunohistochemistry Stainer.

(All steps at room temperature, unless otherwise specified).

- 8. BUF1:** 10 seconds; Rinses the slide cartridge in 500 mls TBS / Tween-20 buffer solution (500 mls TBS buffer and 500 μ l Tween-20, TBS made up as 10 litres stock solution: 80g NaCl, 6.05g TRIS, 44 mls 1M HCl and made up to 10 litres with distilled water, pH adjusted to 7.4 with HCl).
- 9. PAD1:** 45 seconds; Blotting and partial drying of the slides on an absorbent pad.
- 10. HP Block1:** 5 minutes; incubates the slides in Dako Peroxidase Blocking Solution (Dako S2023, hydrogen peroxide solution in phosphate buffer containing NaN₃ and detergent) to block the non-specific tissue action of tissue peroxidases.
- 11. PAD1:** 30 seconds.
- 12. BUF1:** 10 seconds.
- 13. PAD1:** 45 seconds.
- 14. HP Block2:** 5 minutes. Repeats step 3 again.
- 15. PAD1:** 30 seconds.
- 16. BUF1:** 10 seconds.
- 17. PAD1:** 45 seconds.
- 18. HP Block3:** 5 minutes. Repeats step 3 for the third, and final time.
- 19. PAD1:** 30 seconds.
- 20. BUF1:** 10 seconds.
- 21. PAD1:** 30 seconds.
- 22. BUF1:** 10 seconds.

23. PAD1: 30 seconds.

24. BUF1: 10 seconds.

25. PAD1: 30 seconds.

26. BUF1: 10 seconds.

27. PAD1: 45 seconds.

28. AB1: 1 Hour; Applies and incubates the primary antibody on the slides (use Dako mouse monoclonal anti-human androgen receptor immunoglobulin, M3562) at a 1/100 dilution in Dako Antibody diluent (S022).

29. PAD1: 30 seconds.

30. BUF1: 10 seconds.

31. 24 – 31. Repeat **PAD1: BUF1** blotting and rinsing sequence 4 more times.

32. PAD1: 45 seconds.

33. AB2: 30 minutes. Incubates the biotinylated secondary antibody (rabbit anti-mouse antibody, in a buffered solution containing carrier protein and preservative) from Bottle A of the Dako ChemMate® detection kit (Dako K5001).

34. PAD2: 45 seconds; change to a second absorbent pad for slide blotting.

35. BUF2: 10 seconds; change to a 2nd 500 ml reservoir of TBS / Tween-20 buffer solution for these rinses.

36. PAD2: 30 seconds.

37. BUF2: 10 seconds.

38. 38-43. Repeat **PAD2: BUF2** blotting and rinsing sequence 3 more times.

39. PAD2: 45 seconds.

40. HRP: 30 minutes; applies HRP (streptavidin conjugated to horseradish peroxidase in buffered solution containing carrier protein and preservative) from Bottle B in the Dako ChemMate® detection kit (Dako K5001).

41. PAD3: 30 seconds; change to a third absorbent pad for slide blotting.

42. BUF3: 10 seconds; change to a third 500 ml reservoir of TBS / Tween-20 buffer solution for rinsing.

43. Repeat **PAD3: BUF3** blotting and rinsing sequence 4 more times.

44. PAD3: 45 seconds.

45. DAB1: 5 minutes. Applies the chromophore substrate for the horseradish peroxidase (DAB, diaminobenzidene in organic solvent) from Bottles C and D in the Dako ChemMate® detection kit (Dako K5001). Made up as 80 μ l DAB (Bottle C) in 4 mls HRP substrate buffer (Bottle D).

46. PAD4: 30 seconds; change to a fourth absorbent pad used for blotting.

47. BUF2: 10 seconds; rewash in the 2nd 500 ml reservoir of TBS / Tween-20 buffer solution for this wash.

48. PAD4: 45 seconds.

49. DAB2: 5 minutes; repeat the first DAB chromophore step.

50. PAD4: 30 seconds; fourth absorbent pad used for blotting.

51. BUF2: 10 seconds; Rewash in the 2nd 500 ml reservoir of TBS / Tween-20 buffer solution for this wash.

52. PAD4: 45 seconds.

53. DAB3: 5 minutes; repeat the first DAB chromophore step for the third and final time.

54. PAD4: 30 seconds.

55. BUF2: 10 seconds.

56. PAD4: 30 seconds.

57. BUF3: 10 seconds; rewash in the 2nd 500 ml reservoir of TBS / Tween-20 buffer solution for this wash.

58. PAD4: 30 seconds.

59. HAEML: 2 minutes; applies Dako Haematoxylin Solution (S2020) to counterstain any unstained cells for visual contrast on the slides.

60. PAD4: 30 seconds.

61. WATER: 10 seconds.

62. PAD4: 30 seconds.

63. WATER: 10 seconds.

64. PAD4: 30 seconds.

65. When completely dry, mount the specimen on the slide in DPX media (same refractive properties as the coverslip) under a 22 x 50 mm coverslip for analysis.

Appendix 3 Sample Scoring Sheets for Androgen Receptor FISH studies

Patient ID	Patient 06		Batch No:	#21558		Date	04.01	
			Probe:	AR		Observer 1	RB	
AREA 1 56*103.5			AREA 2 55*102			AREA 3 47*101		
	X	AR		X	AR		X	AR
1	1	4	1	2	4	1	1	5
2	1	6	2	1	3	2	1	8
3	1	4	3	1	5	3	1	10
4	1	4	4	1	4	4	1	7
5	1	5	5	1	7	5	1	7
6	1	6	6	2	6	6	1	6
7	1	6	7	1	5	7	1	5
8	1	6	8	1	5	8	1	4
9	1	6	9	1	4	9	1	7
10	1	4	10	1	3	10	1	7
11	1	4	11	1	7	11	1	6
12	2	4	12	1	6	12	1	8
13	2	5	13	1	8	13	1	8
14	1	5	14	1	9	14	1	10
15	1	5	15	1	7	15	1	11
16	1	5	16	1	6	16	1	4
17	1	4	17	1	2	17	1	3
18	1	3	18	1	3	18	1	5
19	1	3	19	1	5	19	1	6
20	1	3	20	1	8	20	1	6
Total	22	92		22	107		20	133

Appendix 3 Sample Scoring Sheets for Androgen Receptor FISH studies (continued)

Patient ID	Patient 06	Batch No:	#21558		Date	04.01		
		Probe:	AR		Observer 2	JE		
AREA 1 56*103.5		AREA 2 55*102		AREA 3 47*101				
	X	AR		X	AR		X	AR
1	1	3	1	1	6	1	1	3
2	1	6	2	1	6	2	1	5
3	1	5	3	1	3	3	1	10
4	1	4	4	1	5	4	1	4
5	1	4	5	1	4	5	2	4
6	1	6	6	1	6	6	1	4
7	1	3	7	1	2	7	1	3
8	1	4	8	1	5	8	2	6
9	1	8	9	1	4	9	1	3
10	1	4	10	1	6	10	1	9
11	1	5	11	1	4	11	1	4
12	1	5	12	1	6	12	1	8
13	1	6	13	1	5	13	1	6
14	1	4	14	1	4	14	1	7
15	1	5	15	1	5	15	1	9
16	1	6	16	2	8	16	2	8
17	1	6	17	1	8	17	1	4
18	1	5	18	1	4	18	1	6
19	1	6	19	1	3	19	1	8
20	1	7	20	1	7	20	1	3
Total	20	102		21	101		23	114

Appendix 3 Sample Scoring Sheets for Androgen Receptor FISH studies (continued)

ID Number:	Patient 06		Batch No:	#21558		
			Date	04.01		
Observer 1		RB	Observer 2	JE		
	X	AR	Ratio	X	AR	Ratio
Area 1	22	92	4.18	20	102	5.10
Area 2	22	107	4.86	21	101	4.81
Area 3	20	133	6.65	23	114	4.96
Mean	Score Obs 1		5.23	Score Obs 2		4.96
Std Dev	Error Obs 1		24.36%	Error Obs 2		2.93%
Overall AR ratio is		5.09	19.55%	0.054	observer error	as a %
						5
Tumour is		Amplified				
Both Observers						
Mean=	5.09		Upper 95% CI	5.80	95% CI= 4.39-5.8	
STD DEV=	0.62		Lower 95% CI	4.39		
Confidence=	0.70					

Appendix 3 Sample Scoring Sheets for Androgen Receptor FISH studies (continued)

Pathology Number:	Patient 06			Batch No:	#21558				
				Date	04.01				
Observer 1:	RB	Per cell		Observer 2:	JE	Per cell		Averages	
	X	AR	X	AR	X	AR	X	X	AR
Area 1	22	92	1.10	4.60	20	102	1.00	5.10	
Area 2	22	107	1.10	5.35	21	101	1.05	5.05	
Area 3	20	133	1.00	6.65	23	114	1.15	5.70	
Mean	Score Obs 1		1.07	5.53	Score Obs 2		1.07	5.28	
Std Dev	Error Obs 1		5.41%	18.75%	Error Obs 2		7.16%	6.85%	

	copy number	error	observer error	as a %
Chromosome X	1.07	1.35%	0	0
AR	5.41	12.70%	0.04622496	5

Both Observers X

Mean= 1.07

Std Dev= 0.0144338

Confidence= 0.016333

Upper 95% CI

1.08

95% CI= 1.05-1.08

Lower 95% CI

1.05

276

Both Observers AR

Mean= 5.41

Upper 95% CI

6.19

95% CI= 4.64-6.19

Std Dev= 0.6866282

Lower 95% CI

4.63

Confidence= 0.7769776

Appendix 4.

Publications and Presentations arising from this work.

Published Papers

Brown R S D, Edwards J, Dogan A, Payne H A, Harland S J, Bartlett J M S, Masters J R W. Amplification of the androgen receptor gene in bone metastases from hormone-refractory prostate cancer. *Journal of Pathology*, 2002;198:237-244.

Brown R S D, Dogan A, Ell P J, Masters J R W, Payne H A, Harland S J. The comparative value of bone marrow aspirate and trephine for obtaining bone scan-targeted metastases from hormone-refractory prostate cancer. *Prostate Cancer and Prostate Diseases* 2002;5:144-151.

Brown R S D, Edwards J, Bartlett J M S, Jones C, Dogan A. Routine acid decalcification of bone marrow samples can preserve DNA for FISH and CGH studies in metastatic prostate cancer. *Journal of Histochemistry and Cytochemistry* 2002;50:113-115.

Oral Presentations

Androgen Receptor Gene Amplification in Bone Metastases from Hormone-Refractory Prostate Cancer. Finalist, Sylvia Lawler Prize Presentation, Royal Society of Medicine, Section of Oncology, 13 March 2002.

The comparative values of bone marrow aspirate and trephine for obtaining bone scan-targeted metastases from hormone-refractory prostate cancer

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Samples of metastatic prostate cancer to bone are difficult to obtain. The aim of this study was to compare the results of bone marrow aspirate and trephine biopsy for obtaining metastatic hormone-refractory prostate cancer (HRPC) samples using previous diagnostic planar 99^{m}Tc -HDP bone scans to guide the procedure. All samples taken were for the purposes of research and molecular studies on HRPC. Twenty patients with HRPC had bone marrow aspirate and trephines taken from lesions in the posterior superior iliac spine or sacro-iliac region when shown on diagnostic 99^{m}Tc -HDP bone scans. Three patients also underwent plain X-ray, 18F-positron emission tomography bone scan, pelvic MRI scan and 99^{m}Tc nanocolloid bone scans. These images were used to assess if the extra imaging information provided, such as three-dimensional localisation of the bone metastases, was of value for target bone metastases. Cancer cells were obtained in 15/20 (75%) cases in which a trephine biopsy was attempted and 0/20 of cases in which a bone marrow aspiration was attempted. The additional information provided by the range of other imaging investigations was of little benefit in obtaining tumour samples, but did suggest why negative biopsies were obtained in some cases after targeting with planar bone scans. We recommend the use of bone marrow trephine biopsy alone, guided by previous diagnostic 99^{m}Tc planar bone scan as a practical method to obtain prostate cancer cells from bone metastases.

Prostate Cancer and Prostatic Diseases (2002) 5, 144–151. doi:10.1038/sj.pca.4500581

Keywords: 99^{m}Tc -bone scan; 99^{m}Tc -nanocolloid bone marrow scan; bone marrow aspiration; bone marrow trephine; Fluorine18 positron emission tomography bone scan; hormone-resistant prostate cancer; pelvic magnetic resonance imaging scan

Introduction

Prostate cancer is a common malignancy amongst men in the Western World. In 1993 in England and Wales 17210 new cases were diagnosed and during the period 1971–1993, the incidence of the disease increased by 179%.¹

Early stage disease may be cured by prostatectomy or radiotherapy but a proportion of these patients will

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relapse with incurable disease. In addition, a significant proportion of patients present with metastatic disease. Bone metastases are a common cause of morbidity and mortality representing a considerable burden for the Health Service. Better therapies for metastatic disease are required. Relatively little is known about the mechanism of the osteoblastic and sclerotic bone metastases produced by prostate cancer. The molecular mechanisms that underlie bone metastases and the development of hormone-insensitive disease at this and other sites need to be understood.

Diagnostic bone marrow aspiration and trephine biopsy are seldom done at disease relapse since the widespread introduction of radionuclide bone scanning. This, together with the difficulty in working with bony samples that contain a mixture of hard and soft tissues² has resulted in bone metastases being relatively understudied (compared with prostatic tissue obtained during therapeutic transurethral resection).

The bone marrow aspirate and trephine samples in this study were obtained for the purpose of molecular studies on androgen receptor immunohistochemical expression, androgen receptor gene amplification and comparative genomic hybridisation studies in hormone-refractory prostate cancer (paper currently in preparation).

This study resulted from two observations. The first was the paucity of published information on our proposed method of targeting bone metastases for biopsy in advanced carcinoma of the prostate using isotope bone scans.³⁻⁶ The second was our observation that bone marrow aspirates from patients with bone metastases from hormone-refractory prostate cancer patients rarely contained cancer cells.

Patients and methods

Patients

Over an 11-month period, eligible patients were recruited from outpatient clinics or during inpatient admission. Hormone-refractory disease was defined as disease progression (requiring a change in therapy) after at least one previous hormonal manipulation and associated with a rising serum prostate specific antigen (PSA). Patients with previous radiotherapy to the sacro-iliac region were excluded from the study on the basis of possible confounding cytogenetic changes due to the radiation.

Methods

The project was given approval by the Local Research Ethics Committee. For all patients the previous history and diagnostic planar Technetium-99^m hydroxymethylene diphosphonate (750 MegaBecquerels 99^mTc-HDP) bone scans (hereafter called bone scan) were reviewed for the presence of bone metastases affecting the sacro-iliac region that would be suitable for biopsy. Bone scans had to have been performed within 1 y. Comprehensive training in bone aspiration and trephine biopsy was given by the haematology service prior to undertaking the collection of study cases. All samples were taken by RB using local anaesthetic at a site selected with direct reference to the bone scan. The posterior superior iliac

spine was located and the site of biopsy chosen with reference to this. Clinical tenderness to palpation was used to help identify metastatic sites if apparent. If the proposed area chosen was not able to be safely biopsied (inaccessible to the aspiration needle), then the posterior superior iliac spine was used instead.

Bone marrow aspirates were taken first, using a 15G MarrowgaugeTM needle (Rocket Medical, Watford, UK). Bone marrow trephine(s) were then taken with a Becton-Dickinson Bone Marrow Biopsy needle (Becton-Dickinson, NJ, USA). Haemostasis was achieved and analgesia given if required.

Diagnostic bone marrow aspirate slides were made using the standard smear technique and sent to the diagnostic haematology service for reporting. Bone marrow trephines were immediately placed in formalin-saline and sent for routine overnight acid decalcification and subsequent histological assessment by the histopathology service. Standard stains and immunohistochemistry (PSA, prostate specific acid phosphatase (PSAP) and the cytokeratin markers CAM 5.2 and MNF116) were performed on all samples. After eight patients had undergone aspirate and trephine biopsies, half of the aspirate sample slides were also sent for immunocytochemistry for PSA and CAM 5.2.

In addition to the standard method of bone scans described, three patients also agreed to have whole body 18-Fluorine (200 MegaBecquerels) positron emission tomography bone scans (hereafter called 18F PET), 99^mTc (375 MegaBecquerels) nanocolloid bone marrow scans (hereafter called nanocolloid bone marrow scan) and MRI scans of the pelvis (including short tau inversion recovery (STIR) sequences for marrow images). The aim of these scans was to either look at the pattern of disease in three dimensions or to look at changes in the bone marrow adjacent to known metastases. A specific ARSAC licence was obtained for the three patients. Plain X-rays of the pelvis were also performed on all patients. All imaging studies were completed prior to each patient undergoing bone marrow aspiration and biopsy.

Spearman log rank test was used to correlate the number of lesions on the bone scan with the serum PSA values.

Results

Twenty of 29 patients approached agreed to participate (69%, see Table 1). One patient had co-existent chronic myeloid leukaemia. The mean age of patients was 71 y and the number of previous treatments for prostate cancer ranged from one to six (covering most curative and palliative modalities used for prostate cancer treatment). Patients had undergone the treatments listed in Table 1 prior to bone marrow aspiration and trephine biopsy. Previous therapy was usually systemic in nature and intended to provide palliation. Only four patients had originally been treated radically. The majority of patients (12/20) had presented with metastatic disease (M1).

All patients had abnormally elevated serum PSA levels; with a range from 19 to 3306 ng/ml. Anaemia was a common finding on the full blood count (FBC)

Table 1 Clinical characteristics of bone marrow aspiration and trephine patients

Patient number	Age	Stage at first presentation ^a	Matching primary tumour	Previous treatments for prostate cancer
1	76	M1	N	O, S & HC
2	65	T1C	N	MAB, HC, S
3	70	T3M1	N	MAB
4	70	M1	N	G, S & HC
5	78	M1	N	TUR & O, F, S & HC
6	78	M1	Y	TUR, MAB, S & HC, Mitox
7	69	M1	Y	O, CPA
8	75	pT3 N0	Y	RP, O, F, S & HC
9	70	M1	N	O, F, S, ECarboF, Mitox, 5-FU/FA
10	74	M1	Y	G, Zoled, F
11	65	pT3b	Y	RP, G & CPA, B
12	72	M1	N	O, CPA, Z, S, B, Mitox
13	62	M1	N	MAB
14	60	M1	N	G, Estra, S & HC
15	75	M0	Y	TUR, Zoled
16	73	M0	Y	RT & G, S & HC, B
17	62	M1	N	G, B, P, ECarboF, S
18	64	T2 N0	N	RT, MAB, S & HC
19	66	M0	Y	TUR, G
20	88	M1	N	G
FBC at time of biopsy				
Number of hot spots on bone scan	PSA at time of biopsy	Hb	WBC	Platelets
1	11	47	13.5	14.6
2	27	95	11.3	4.0
3	7	267	13.4	7.4
4	18	69	10.6	6.5
5	58	27	13.8	11.0
6	42	3064	11.6	5.1
7	21	276	11.5	7.2
8	53	19	13.6	9.4
9	31	398	12.2	10.4
10	32	45	15.9	9.8
11	8	29	14.0	9.7
12	18	3306	12.7	8.8
13	Superscan	311	10.5	10.5
14	42	111	8.3	3.6
15	38	182	12.2	9.3
16	47	120	11.5	9.9
17	73	1730	11.5	7.6
18	53	41	12.4	6.1
19	11	375	10.0	2.6
20	29	133	9.8	2.9

^a1997 UICC T N M Stage.

B: bicalutamide, CPA: cyproterone acetate, Estra: estramustine, ECarboF: epirubicin, carboplatin and 5-FU, F: flutamide, 5-FU/FA: 5-fluorouracil and folinic acid, G: Goserelin, HC: hydrocortisone, MAB: maximum androgen blockade, Mitox: mitoxantrone, N: no, O: orchidectomy, P: prednisolone, S: stilbestrol, RP: radical prostatectomy, RT: radical radiotherapy, TUR: transurethral resection, Y: yes, Zoled: Zoledronate.

with 14/20 patients having a haemoglobin below the laboratory reference range of 13 g/dl. However, only 3/20 patients had a white cell count (WBC) below $4 \times 10^9/l$ and only a single patient had an abnormally low platelet count (patient 10, $114 \times 10^9/l$). Two patients with a low WBC also had a low haemoglobin level suggesting possible marrow failure, but in both cases the bone marrow aspirates were negative and the trephine biopsies showed less than a 5% tumour cell infiltrate. The range of lesions visible on the AP planar bone scans for all patients ranged from seven to over 50. In addition, one patient had a 'superscan' (widespread tracer uptake with no renal tracer excretion visible at the time of scanning). The two patients with blood counts suggestive of some marrow failure did not have very high hot spot counts on the bone scans. We could find no correlation between the level of serum PSA and the number of hot spots on the bone scan ($r=0.12$, Spearman log rank test).

The results of the aspiration and biopsies are shown in Table 2. In two cases no material was obtained due to a dry tap on aspiration and a failed trephine biopsy, secondary to sclerotic bone (unable to be penetrated by the trephine needle). In the remaining 18 cases, a suitable bone marrow trephine was obtained. For three of the 18 trephine biopsies (17%) a normal marrow sample with no evidence of tumour deposits was obtained. Fifteen of the 18 successful trephines (83%) contained metastatic tumour. In one of these samples only a single microscopic focus of disease was seen on immunohistochemistry.

Of 18 attempts at bone marrow aspiration, no material was aspirated in six due to a dry tap. These failures were associated with either diffuse tumour infiltration in the trephine biopsy (4/6) or also had an unsuccessful trephine biopsy (2/6). Ten of the aspirates contained sufficient particulate material to assess. In one case

Table 2 Results of bone marrow aspirates and trephines

Patient number	Bone marrow aspirate	Bone marrow trephine
1	Negative, normal lineages	Positive, multiple tumour foci
2	Normal lineages, ? occasional suspicious cells	Negative
3	Negative, normal lineages	Positive (10% of marrow space)
4	Dry Tap	Failed
5	Negative, normal lineages	Negative
6	Dry Tap	Positive, Diffuse infiltration
7	Negative, haemodilute	Positive, 1 focus
8 ^b	Negative, normal lineages	Positive, 1 focus
9 ^{a,b}	Negative, normal lineages	Negative
10 ^{a,b}	Negative, normal lineages	Positive, multiple tumour foci
11 ^a	Negative, haemodilute	Positive, multiple tumour foci, known CML
12 ^a	Dry Tap	Positive, Diffuse tumour infiltration
13 ^a	Dry Tap	Failed
14 ^a	Dry Tap	Positive, Diffuse tumour infiltration
15 ^a	Dry Tap	Positive, Diffuse tumour infiltration
16 ^a	Negative, normal lineages	Positive, multiple tumour foci
17 ^a	Negative, erythroid dysplasia	Positive, multiple tumour foci
18 ^a	Negative, normal lineages	Positive, multiple tumour foci (25% of marrow space)
19 ^a	Haemodilute, ? occasional suspicious cells	Positive, multiple tumour foci (< 5% of marrow space)
20 ^a	Negative, haemodilute	Positive, multiple tumour foci (< 5% of marrow space)

CML: chronic myeloid leukaemia.

^aFor the last 11 patients bone marrow aspirate slides (if obtained) were also sent for immunocytochemistry; in no instances did they show evidence of tumour cells.^bThe three patients who underwent multiple bone imaging for assessment.

suspicious cells were seen, but this was associated with a normal trephine biopsy. In the remaining cases no evidence of tumour cells was seen. Four aspirate samples were haemodilute and sub optimal for assessment. One of these was reported as containing possible occasional suspicious cells but the trephine showed low levels of tumour infiltrate (< 5%).

Of nine cases where aspirate slides were also sent for immunocytochemistry (PSA and CAM 5.2), no evidence of tumour infiltrates were seen, in complete agreement with the haematological assessment.

The bone marrow sampling was generally well tolerated by patients with no major complications. One patient had a pre-syncope episode that settled with a short period of bed rest after the procedure. The majority of patients were already on compound analgesics, non-steroidal anti-inflammatory drugs or opiates because of bone metastases prior to the aspirate and trephine biopsies. Local pain after the procedure was uncommon and patients seldom required extra analgesia. In three out of the 20 cases the patient would have declined to undergo a similar procedure again, two of these cases were in patients with a failed aspirate and trephine due to sclerotic bone.

For the three patients who underwent the other types of bone imaging (plain X-ray, 18F PET bone scan, nanocolloid bone marrow scans and MRI scans of the pelvis), the target lesion was successfully identified in all three patients on the PET bone scans and MRI scans of the pelvis, as in Figure 1B and C. In no case did the nanocolloid bone marrow scan identify the target lesions in the sacro-iliac region (see Figure 2A). In only one patient did the nanocolloid bone marrow scan identify any bone marrow abnormality (a solitary accumulation defect which matched a known hot spot on the diagnostic bone scan, data not shown). Of the three plain X-rays taken, an abnormality at the site of the target lesion was seen in one case (data not shown).

The corresponding nanocolloid bone marrow scan and pelvic X-ray for the patient in Figure 1 are shown in Figure 2.

Discussion

The detection of metastases within bone marrow by needle aspiration was first reported in 1936 and included a patient with metastatic carcinoma of the prostate.⁷ Specific studies on the bone marrow changes in prostate cancer then began to appear in the 1940s and 1950s.^{8,9} Early studies were mainly in the context of diagnosis and staging, particularly with reference to diagnosing occult bone metastases radiologically, and to try and prevent unnecessary radical surgery.¹⁰ As new diagnostic techniques became available, such as improvements in chemical pathology (eg bone marrow acid phosphatase)⁴ histopathology techniques (eg immunohistochemistry)¹¹ and molecular biology (eg polymerase chain reaction)¹² further studies have continued to be published.

Bone and bone marrow samples are inherently more difficult to work with than some other tissue types, due to the mixture of hard and soft material and the need for acid or EDTA decalcification. Tissue such as prostate gland, removed at transurethral resection, is justified on diagnostic and therapeutic grounds, as well as providing material for study. Bone marrow aspiration and trephine biopsy is no longer a standard diagnostic procedure in prostate cancer. Varenhorst *et al* showed that bone scanning was superior to random bone marrow aspiration in staging prostate cancer in 1983.¹³

Bone scans are the most sensitive bone imaging technique our patients routinely undergo, and bone marrow aspiration and trephine can be done by a trained individual without the aid of interventional radiology. The combined use of bone marrow aspiration and trephines that had been targeted by previously performed bone

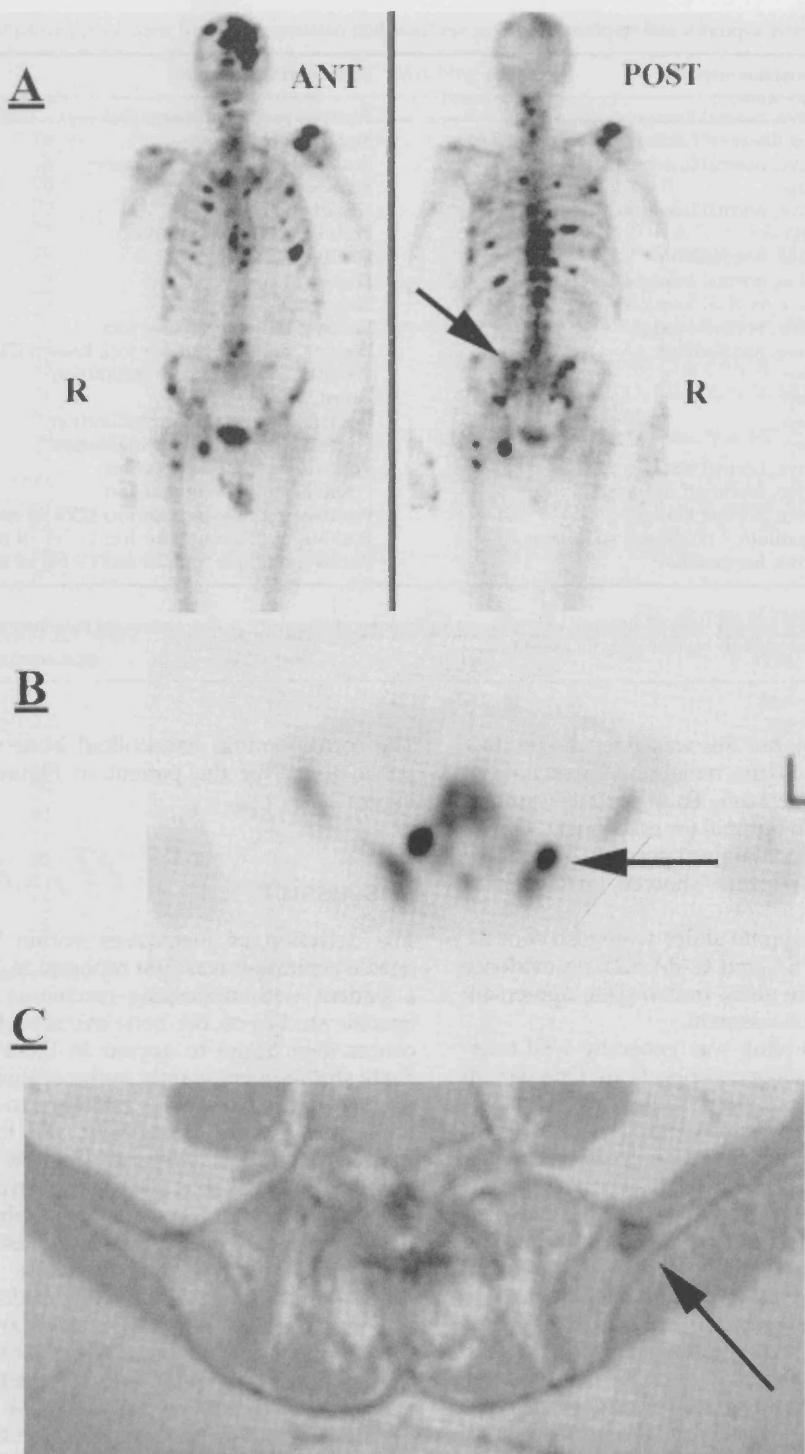


Figure 1 Bone scan, PET bone scan and pelvic MRI of patient 8 in whom we were unable to obtain useful tissue for molecular biology studies. (A) 99^{m}Tc -bone scan with targeted lesion shown by the arrow. (B) 18^{F} PET bone scans (axial view) of the pelvis showing the position of the targeted lesion. (C) Axial pelvic MRI view of the target lesion.

scans appeared to be a good approach for obtaining cancer cells for research studies, but no published information on the success rates in prostate cancer bone metastases was available.

This method yielded a high sampling rate for trephine biopsies (15/20 attempted cases, 15/18 submitted tre-

phines positive), but no yield for aspiration. It is known that trephine biopsy is more sensitive than bone marrow aspiration for the detection of metastatic disease in the bone and bone marrow.¹⁴ However, there is considerable variation within the reported literature on the positive aspiration rate for bone marrow aspiration. The older

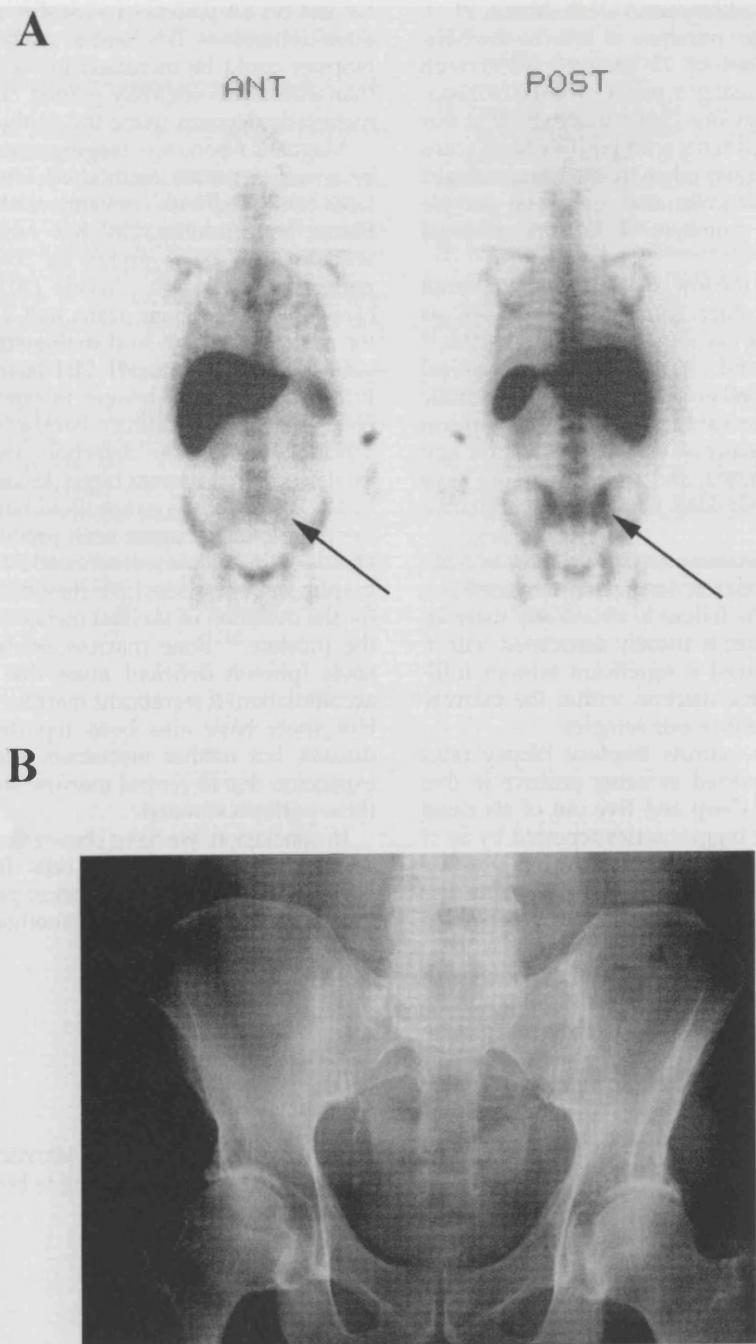


Figure 2 Further images from the same patient as Figure 1. (A) 99^{m} Tc-nanocolloid bone marrow scan showing no evidence of an accumulation defect in the area of the sacro-iliac region (or other sites). (B) Plain X-ray of the pelvis showing no definite abnormalities at the site of the lesion visible on bone scan, PET bone scan or MRI.

literature on prostate cancer bone metastases reports positive aspiration rates between 7 and 50% for mixed groups of early and advanced prostate cancer cases (although the majority of positive aspirates were in patients with known bone metastases in all studies).^{8-10,15,16} Early studies did not have the benefit of modern techniques such as immunocytochemistry. The use of immunocytochemistry did not increase the positive marrow aspiration rate in smears of the nine cases

assessed here. In micrometastases from breast cancer, immunocytochemical methods can detect tumour cells in marrow/tumour mixtures down to 0.00025%.¹⁷ Positive bone marrow aspiration rates using morphology assessment in patients with known bone metastases from breast cancer show a significant proportion of tumour cells in the aspirate (40%).¹⁸ In prostate cancer patients with known M1 disease, positive aspiration rates varied between 20 and 58% when random biopsies were

performed in the older literature.^{8,9,15,16} Mansi *et al* reported moderate to large numbers of cells in the bone marrow aspirate of 11 out of 15 patients (73%) with known bone metastases using a panel of immunohistochemical markers.¹¹ A previous preliminary study at our centre of prostate cancer patients with positive bone scans showed three of nine aspirates taken by the haematologist contained prostate cancer cells, and only one sample contained cells in large numbers (T Begum, personal communication).

It is difficult to explain the low aspiration rate reported here in comparison to other studies. Tumor load, as indirectly assessed by the combination of serum PSA,¹⁹ bone scan lesion count²⁰ and FBC results, does not reveal results different to many other patients seen in the clinic with progressive prostate cancer. We did not perform reverse transcriptase polymerase chain reactions on any of the blood samples obtained, and therefore do not have data on circulating tumour load in a more quantitative fashion.

One reason for lower marrow aspiration rates in bone metastases from prostate cancer (and other tumours) has long been recognised.⁸ The failure to obtain any material on bone marrow aspiration is usually associated with a trephine biopsy that showed a significant tumour infiltrate and a marked fibrotic reaction within the marrow space. This occurred in four of our samples.

Positive random bone marrow trephine biopsy rates have been previously reported as being positive in five out of 14 cases (36%) by Crisp and five out of six cases (83%) by Spiers.³⁻⁶ In the biggest series reported by Sy *et al*, 32 out of 41 patients (78%) with advanced prostate cancer had positive random iliac bone biopsies. In the only other study of prostate cancer patients reporting targeted biopsies, Chua *et al* obtained 18 out of 19 (95%) positive trephine biopsies in a group of patients with known bone metastases targeted by X-ray guidance (as part of a mixed series of random and image-guided bone marrow biopsies).³

One reason for the failure of bone marrow trephine biopsy to obtain metastases (apart from significant osteosclerosis) is shown in Figures 1 and 2. Without image-guided biopsies small lesions (such as those demonstrated) may be difficult to target accurately by clinical sampling alone. The standard diagnostic bone scan used to guide all our biopsies were two-dimensional planar (anteroposterior) images. The absence of information on the depth of the targeted lesion may limit the successful sampling of smaller lesions. Such depth information can be obtained from bone scans by using a tomographic technique on a diagnostic gamma camera (producing images that are very similar to the axial PET view shown in Figure 1B). Such views are rarely used for diagnostic scans in prostate cancer patients where the information required usually concerns the question are there bone metastases present or not, or have known bone metastases changed over time.

Magnetic resonance imaging of the pelvis and PET bone scanning can also provide information on target lesion depth (with MRI able to be used to measure an accurate depth, Figure 1C). When there was only a small target lesion, the use of extra information on depth still did not allow a successful biopsy sample to be taken. These imaging modalities are also not routinely available

for use on all patients to identify the site of disease in three-dimensions. It is hard to see how the rate of positive biopsies could be increased further using clinical rather than direct radiologically guided biopsies to target small metastatic deposits using the method described.

Magnetic resonance imaging scans have been shown to be a very sensitive method of detecting skeletal metastases compared with conventional bone scintigraphy.^{21,22} Planar bone scintigraphy has recently been compared with 18F PET bone studies by Schirrmeister *et al* in 44 patients with known prostate (20/44), lung or thyroid carcinoma.²³ The bone scans had a decreased sensitivity for detecting benign and malignant lesions in the bony pelvis compared with 18F PET bone scans. The three 18F PET and pelvic MR images in our study were however not performed for direct comparison of sensitivity or specificity for lesion detection, but rather for further localisation of a known target lesion.

The results of the nanocolloid bone marrow studies on the three patients agree with previously published data. Haddock *et al* reported nanocolloid bone marrow scintigraphy to be less sensitive than conventional bone scans for the detection of skeletal metastases from carcinoma of the prostate.²⁴ Bone marrow scans usually reveal cold spots (photon deficient areas due to defects in tracer accumulation) if significant marrow infiltration is present. Hot spots have also been reported due to metastatic disease, but neither metastases nor peripheral marrow expansion due to central marrow failure were seen in the three patients scanned.

In conclusion, we have shown that trephine biopsy can be used to obtain cancer cells from the majority of hormone-resistant prostate cancer patients using previous diagnostic bone scans as a method for targeting bone deposits.

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References

- 1 Majeed A, Babb P, Jones J, Quinn M. Trends in prostate cancer incidence, mortality and survival in England and Wales 1971-1998. *BJU Int* 2000; **85**: 1-5.
- 2 Alers JC, Kritjenberg P-J, Vissers KJ, van Decken H. Effect of bone decalcification procedures on DNA in situ hybridization and comparative genomic hybridization: EDTA is highly preferable to a routinely used acid decalcifier. *J Histochem Cytochem* 1999; **47**: 703-709.
- 3 Chua DT, Ackermann A, Veenema RJ. Bone marrow biopsy in patients with carcinoma of the prostate. *J Urol* 1969; **102**: 602-606.
- 4 Sy FA, Gursel EO, Veenema RJ. Positive random iliac bone biopsy in advanced prostate cancer. *Urology* 1973; **11**: 125-127.
- 5 Crisp J. Random bone marrow biopsy in the staging of carcinoma of the prostate. *Br J Urol* 1976; **48**: 265-267.

6 Spiers ASD, Deal DR, Kasimis BS, Miller BR. Evaluation of the bones and bone marrow in patients with metastatic carcinoma of the prostate: radiological cytologic and cytogenetic findings. *J Med* 1982; **13**: 303–307.

7 Rohr K, Hegglin R. Tumorzellen im sternalpunktat. *Deutsch Arch klin Med* 1936; **179**: 61–79.

8 Alyea EP, Rundles RW. Bone marrow studies in carcinoma of the prostate. *J Urol* 1949; **62**: 332–339.

9 Clifton JA, Philipp RJ, Ludovic E, Fowler WM. Bone marrow and carcinoma of the prostate. *Am J Med Sci* 1952; **224**: 121–130.

10 Welch JF, Mackinnery CC. Experience with aspiration biopsies of the bone marrow in the diagnosis and prognosis of carcinoma of the prostate gland. *Am J Clin Pathol* 1964; **41**: 509–512.

11 Mansi JL et al. Detection of tumour cells in bone marrow of patients with prostatic carcinoma by immunocytochemical techniques. *J Urol* 1988; **139**: 545–548.

12 Wood Jr DP et al. Identification of bone marrow micrometastases in patients with prostate cancer. *Cancer* 1994; **74**: 2533–2540.

13 Varenhorst E, Alund G, Lindstrom E, Manson JC. Bone marrow aspiration biopsy and bone scanning in the staging of prostate cancer. *Br J Urol* 1983; **55**: 534–537.

14 Bearden JD, Ratkin GA, Coltman CA. Comparison of the diagnostic value of bone marrow biopsy and bone marrow aspiration in neoplastic disease. *J Clin Pathol* 1974; **27**: 738–740.

15 Mehan DJ, Broun GO, Hoover B, Storey G. Bone marrow finding in carcinoma of the prostate. *J Urol* 1966; **95**: 241–244.

16 Nelson CMK, Boatman DL, Flocks RH. Bone marrow examination in carcinoma of the prostate. *J Urol* 1973; **109**: 667–670.

17 Molino A et al. A comparative analysis of three different techniques for the detection of breast cancer cells in bone marrow. *Cancer* 1991; **67**: 1033–1036.

18 Ingle JN, Tormey DC, Tan HK. The bone marrow examination in breast cancer; diagnostic considerations and clinical usefulness. *Cancer* 1978; **41**: 670–674.

19 Grossmann ME, Huang H, Tindall DJ. Androgen receptor signalling in androgen-refractory prostate cancer. *J Natl Cancer Inst* 2001; **93**: 1687–1697.

20 Soloway MS et al. Stratification of patients with metastatic prostate cancer based on extent of disease on initial bone scan. *Cancer* 1988; **61**: 195–202.

21 Steinborn MM et al. Whole body bone marrow MRI in patients with metastatic disease to the skeletal system. *J Comput Assist Tomogr* 1999; **23**: 123–129.

22 Algra PR et al. Detection of vertebral metastases: comparison between MR imaging and bone scintigraphy. *Radiographics* 1991; **11**: 219–232.

23 Schirrmeister H et al. Sensitivity in detecting osseous lesions depends on anatomical localization: planar bone scintigraphy versus ¹⁸F PET. *J Nucl Med* 1999; **40**: 1623–1629.

24 Haddock G et al. ^{99m}Tc-Nanocolloid bone marrow scintigraphy in prostate cancer. *Br J Urol* 1989; **63**: 497–502.

BRIEF REPORT

Routine Acid Decalcification of Bone Marrow Samples Can Preserve DNA for FISH and CGH Studies in Metastatic Prostate Cancer

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SUMMARY Production of paraffin-section material from tissue samples that contain bone requires decalcification. Techniques such as acidic decalcification or EDTA chelation are suitable methods. Acid decalcification is generally quicker than EDTA chelation but studies have suggested that it may result in hydrolysis of DNA. Here we show that limited acid decalcification (less than 24 hr) in 5% formic acid can preserve DNA sufficient for fluorescent *in situ* hybridization (FISH) or comparative genomic hybridization (CGH) and that prolonged 10% formic acid decalcification results in failure of FISH and only limited retrieval of DNA for CGH studies. (*J Histochem Cytochem* 50:113–115, 2002)

KEY WORDS

bone marrow
comparative genomic
hybridization (CGH)
decalcification
DNA
formic acid
fluorescent *in situ* hybridization
(FISH)

Routine acid decalcification (RDO; Apex Engineering Products, Plainfield, IL) for bone marrow trephine and autopsy bone samples has been reported to result in failure to obtain DNA for *in situ* hybridization (ISH), comparative genomic hybridization (CGH), and flow cytometric studies in trephine and autopsy bone marrow samples from prostate cancer (Alers et al. 1999). Decalcification using 10% EDTA for three autopsy bone marrow specimens resulted in successful FISH, CGH, and flow cytometric studies and gave better preservation of architecture for routine staining and immunohistochemistry. The authors concluded that EDTA was highly preferable to their routinely used acid decalcified bone where studies on DNA were required.

We also routinely use acid decalcification for bone marrow trephine specimens (12–18 hr) but use a 5% formic acid (Becton Dickinson Laboratory Supplies; Mountain View, CA) solution in distilled water rather than a hydrochloric acid-based product (RDO). This method of decalcification has enabled us to success-

fully perform FISH on 15 of 15 samples analyzed for androgen receptor (AR) gene copy number in bone metastases from prostate cancer (SpectrumOrange AR FISH probe, SpectrumGreen centromere FISH probe for the X chromosome; Vysis, Downers Grove, IL) (see Figure 1A). AR gene amplification has been implicated in the development of hormone-refractory prostate cancer (HRPC), being found in approximately 30% of locally recurrent hormone-refractory biopsies and rarely in hormone-naïve specimens (Visakorpi et al. 1995). A similar figure has been reported for the prevalence of AR gene amplification in distant metastases from HRPC (Bubendorf et al. 1999), but to date no studies have looked at AR gene amplification in bone metastases. This was the primary aim of our study. Amplification of the AR gene was found in 5/12 (38%) of bone metastases (in the remaining three cases, scoring for AR gene copy number in tumor cells was not performed because of a low number of malignant cells in the trephine biopsies).

The FISH studies on decalcified trephine material required an additional pretreatment, i.e., microwave antigen retrieval technique, before standard tissue digestion with pepsin (M. Farquharson, Department of Pathology, Glasgow Royal Infirmary; personal communication).

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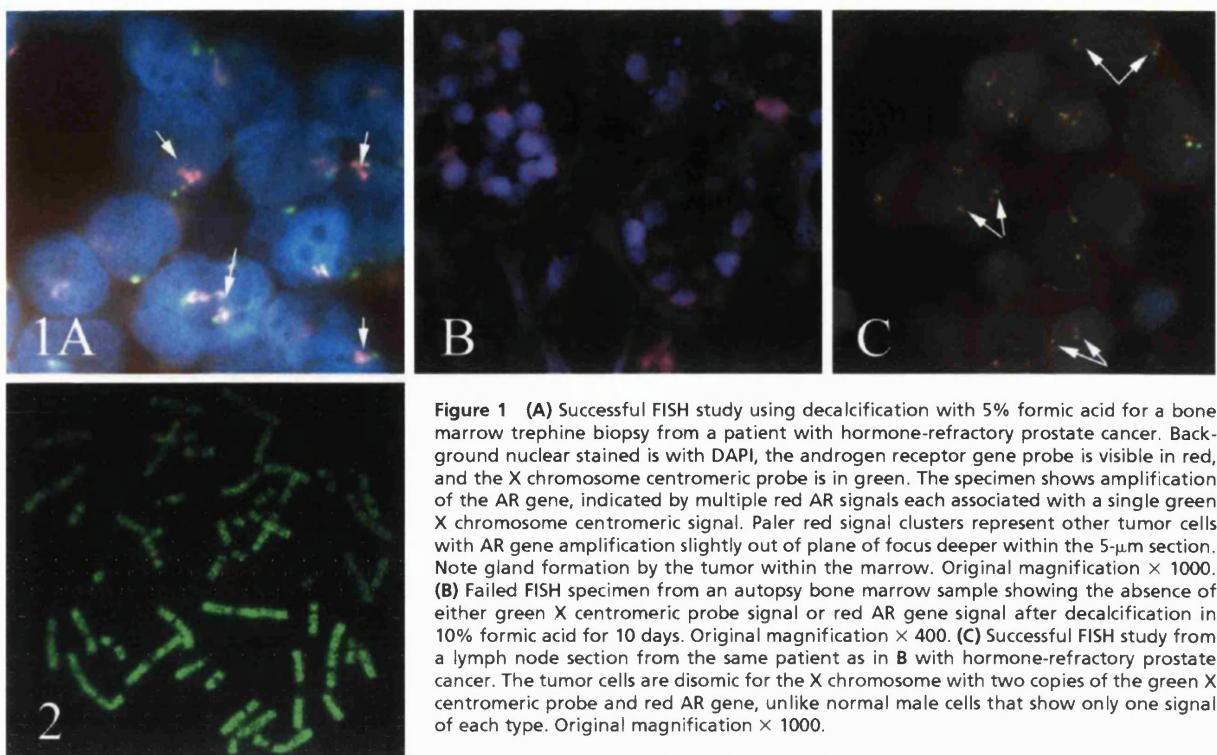


Figure 1 (A) Successful FISH study using decalcification with 5% formic acid for a bone marrow trephine biopsy from a patient with hormone-refractory prostate cancer. Background nuclear stained with DAPI, the androgen receptor gene probe is visible in red, and the X chromosome centromeric probe is in green. The specimen shows amplification of the AR gene, indicated by multiple red AR signals each associated with a single green X chromosome centromeric signal. Paler red signal clusters represent other tumor cells with AR gene amplification slightly out of plane of focus deeper within the 5- μ m section. Note gland formation by the tumor within the marrow. Original magnification $\times 1000$. (B) Failed FISH specimen from an autopsy bone marrow sample showing the absence of either green X centromeric probe signal or red AR gene signal after decalcification in 10% formic acid for 10 days. Original magnification $\times 400$. (C) Successful FISH study from a lymph node section from the same patient as in B with hormone-refractory prostate cancer. The tumor cells are disomic for the X chromosome with two copies of the green X centromeric probe and red AR gene, unlike normal male cells that show only one signal of each type. Original magnification $\times 1000$.

Figure 2 Hybridization of extracted tumor DNA to normal metaphase chromosomes for comparative genomic hybridization.

Formic acid decalcification has also enabled us to successfully hybridize extracted tumor DNA from decalcified bone marrow trephines to normal metaphase chromosomes in five cases studied thus far (Figure 2) for CGH. Degenerate oligonucleotide primer (5'-CGACTCGAGNNNNNNATGTGG-3') polymerase chain reaction (DOP-PCR) products of up to 1500 base pairs in length have been generated from all 15 decalcified trephine samples (Figure 3).

For larger autopsy bone marrow specimens, we use a 10% formic acid solution for decalcification over a 7–10-day period (with replenishment of the solution on one to two occasions during this period). Similarly to Alers et al. (1999), we found that in five autopsy bone marrow specimens studied FISH could not be successfully performed, whereas studies on soft tissue samples from the same autopsy cases were successful (Figures 1B and 1C). Unlike the cases reported by Alers et al., we have been able to produce DNA fragments using DOP-PCR of up to 1500 base pairs suitable for CGH in two of five of autopsy cases studied (Figure 3).

Little published information is available on the effects of formic acid decalcification on DNA degradation. We have been unable to find other reports of

successful studies using FISH or CGH on formic acid-decalcified bone marrow trephine biopsies (Medline Search 1966–2001). Sarsfield et al. (2000) have reported that formic acid decalcification of bone marrow trephines degrades DNA for PCR using specific primers. In this comparative study of formic acid and EDTA decalcification in 11 bone marrow trephine specimens, similar quantitative amounts of DNA could be retrieved using both decalcification methods, but formic acid pretreatment resulted in a degraded DNA smear and failure to obtain specific PCR products in the majority of samples tested. Provan et al. (1992) were also able to generate a 294-base pair PCR product in only six of ten after formic acid decalcification of paraffin-embedded bone marrow biopsies. By contrast, EDTA decalcification resulted in successful amplification of specific PCR products up to 643 bp in the study by Sarsfield et al. (2000), and the author's previous experience of EDTA for decalcification of bone marrow trephines alone strongly supports this finding (Wickham et al. 2000).

We conclude that routine acid decalcification with 5% formic acid can satisfactorily preserve DNA for some types of molecular biological studies (FISH and CGH) on prostate cancer bone metastases taken by

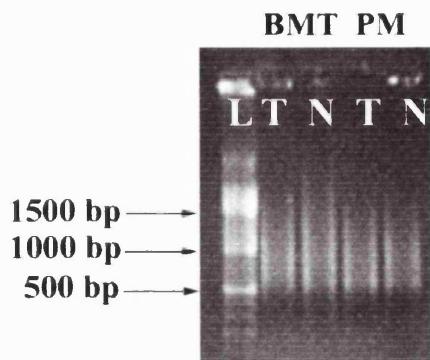


Figure 3 Agarose gel electrophoresis of DOP-PCR DNA fragments after extraction and formic acid decalcification of bone marrow biopsies. BMT, 5% formic acid-decalcified bone marrow trephine biopsy; PM, 10% formic acid decalcification of an autopsy bone sample; T, tumor; N, normal tissue; L, DNA base pair ladder.

trephine biopsy. Longer and stronger acid decalcification resulted in unsuccessful FISH in all autopsy specimens, whereas non-calcified material from the same postmortem samples worked satisfactorily. Decalcification using EDTA appears to offer the best chance of successful DNA retrieval from bone tissue and appears to be the method of choice for decalcification in a prospective study of bone marrow trephine and autopsy samples. Five percent formic acid decalcification avoids major acid hydrolysis of DNA, as judged by

our results with FISH and CGH studies on decalcified trephine material. Attempts to use techniques such as FISH and CGH on archival material should not automatically be abandoned if retrospectively collected specimens have been decalcified in formic acid.

Literature Cited

Alers JC, Krijtenberg P-J, Vissers KJ, van Dekken H (1999) Effect of bone decalcification procedures on DNA in situ hybridization and comparative genomic hybridization: EDTA is highly preferable to a routinely used acid decalcifier. *J Histochem Cytochem* 47:703-709

Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, Kallioniemi OP (1999) Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence in situ hybridization on tissue microarrays. *Cancer Res* 59:803-806

Provan AB, Hodges E, Smith AG (1992) Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. *J Clin Pathol* 45:763-765

Sarsfield P, Wickham CL, Joyner MV, Ellard S, Jones DB, Wilkins BS (2000) Formic acid decalcification of bone marrow trephines degrades DNA: alternative use of EDTA allows the amplification and sequencing of relatively long PCR products. *Mol Pathol* 53:336

Visakorpi T, Hytyinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP (1995) In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nature Genet* 9:401-406

Wickham CL, Boyce M, Joyner MV, Sarsfield P, Wilkins BS, Jones DB, Ellard S (2000) Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *Mol Pathol* 53:19-23

Original Paper

Amplification of the androgen receptor gene in bone metastases from hormone-refractory prostate cancer

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Abstract

The aim of this study was to examine the prevalence of androgen receptor (AR) amplification in metastases to bone and other sites in patients with hormone-refractory prostate cancer (HRPC) and to compare these findings with those in pretreatment primary tumour samples from the same patients. Tissue from 24 patients with HRPC was available for study, together with 13 primary tumour specimens. AR gene amplification and copy number for X-chromosome were assessed by fluorescence *in situ* hybridization (FISH) using a SpectrumOrange™-labelled probe at locus Xq11–13 for the AR gene and a SpectrumGreen™-labelled alpha-satellite probe for the X-chromosome (Vysis, UK, Ltd.). A minimum of 20 nuclei were scored in each of three tumour areas by two independent observers. Samples from 18/24 patients with HRPC (12 bone marrow biopsies, three local tumour recurrences, and three lymph nodes) and nine primary tumour specimens were adequate for FISH analysis. Results were expressed as a mean ratio of AR gene copy number : mean X-chromosome number, with a ratio of greater than 1.5 defined as amplification. AR gene amplification was seen in 9/18 (50%) cases of HRPC and in none of the primary (untreated) tumour specimens ($p = 0.0048$, Fisher's exact test). For the 12 bone marrow samples, AR gene amplification occurred in 5/12 (38%) cases. Elevated copy number for chromosome X occurred in 3/18 (17%) HRPC and 4/9 (44%) matched primary tumours. This study shows for the first time that AR gene amplification can be demonstrated by FISH in bone metastases from HRPC patients. Because bone marrow biopsies can be obtained from most patients with HRPC, the findings provide a rational basis for the routine selection of patients who may respond more favourably to second-line anti-androgen therapy.

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Introduction

Prostate cancer is one of the leading causes of death from malignancy in men in the western world [1]. For men with advanced or metastatic disease, cure is unlikely and hormone therapy is the mainstay of treatment. Despite a high initial response rate of up to 80%, relapse and progression to hormone-refractory disease occur with a median time of 18 months [2]. Currently there is no effective treatment for hormone-refractory prostate cancer (HRPC) and median survival for this stage of the disease is approximately 6 months [3]. Bone metastases are present in almost all men with metastases from HRPC [4] and are a common cause of morbidity and mortality. A better understanding of the mechanisms of progression to hormone-refractory disease may lead to improved treatment outcome at this and other sites of metastatic disease.

Androgen receptor (AR) protein is expressed in nearly all primary and metastatic prostate cancers [5]. Amplification of the AR gene, together with gain of chromosome X (increase in copy number of AR and X centromeric regions), was first reported in 1995 by Visakorpi *et al.* and suggested as a possible mechanism for progression of human prostate cancer following hormone therapy [6]. The precise mechanism by which AR gene amplification is related to the development of HRPC remains unknown. Amplification of genetic material is a well-recognized method of up-regulating gene expression and HRPC cells in which the AR gene is amplified may be more efficient in utilizing the low levels of circulating androgens still present despite hormone-deprivation therapy, resulting in a selective growth advantage [7].

Amplification of the AR gene in HRPC has been confirmed [6–14]. Amplification rates in the region of 15–30% are typical, although the criteria for

amplification have varied between studies. These studies mainly describe locally recurrent disease, two including soft tissue metastases [9,10]. No previous study reported AR gene amplification rates from bone metastases. Bone metastases have been considered difficult to work with due to the mixture of hard and soft tissue [15]. Nevertheless, bone marrow samples can be obtained from most patients [16].

AR gene amplification following initial androgen deprivation monotherapy predicts response to maximum androgen blockade as second-line therapy [12]. It was suggested that clinical studies to evaluate AR gene amplification should ideally be done on metastatic disease, such as bone metastases, rather than local recurrences [12]. If it is possible routinely to diagnose a subgroup of HRPC patients with AR gene amplification in bone metastases, such patients could be treated with the expectation of better response rates.

To investigate the prevalence of AR gene amplification and aneusomy of chromosome X in bone metastases and other samples from HRPC patients, we studied formalin-fixed tissue from bone marrow trephines, local recurrences, distant nodal metastases, and matching primary tumour samples.

Patients and methods

Patient material

The project was given approval by the Local Research Ethics Committee. All patients in the study had HRPC, defined as a rising serum PSA, sufficient to change the clinical management after at least one hormone manoeuvre designed to reduce circulating androgen levels. Suitable patients from the oncology wards or outpatient clinics of our hospital were approached for agreement to participate in the study and informed consent was obtained. Tissue from 24 patients was available for analysis. The samples consisted of 15 bone marrow trephine biopsies, five autopsy samples (bone and soft tissue), three local tumour recurrences, and a metastasis in a supraclavicular lymph node. Primary tumours were either prostate biopsies or radical prostatectomy specimens. The post-mortem samples were obtained from the histopathology department during the period of the study, with consent for the material to be used for research being obtained at the time of consent for the post-mortem.

FISH

The FISH protocol used for soft tissue samples is reported in a previous paper [14]. Briefly, 5- μ m formalin-fixed, paraffin-embedded tissue sections on silane slides were dewaxed, rehydrated, and processed in 0.2 N HCl, followed by exposure to 9% sodium thiosulphate at 80 °C. Tissue digestion was performed with 0.05% pepsin at 37 °C for 26 min, followed by post-fixation in 10% formalin and finally dehydration through an alcohol series. All these steps were carried

out using a VP2000 robotic slide processor (Vysis, UK, Ltd.).

For bone samples, after decalcification in 5% formic acid for bone marrow trephine samples or 10% formic acid for post-mortem bone samples, a similar pre-treatment protocol was undertaken manually. Modifications included the replacement of the tissue permeability (0.02 N HCl) and reducing agent (sodium thiocyanate) steps with a customized antigen retrieval process (personal communication, M. Farquharson, Department of Pathology, Glasgow Royal Infirmary). Here, a pressure container (Menerini Diagnostics, UK) containing 1 l of retrieval buffer (1 l of distilled water, 0.37 g of EDTA, 0.55 g of Trisma base, pH 8.0) was preheated (15 min at full power) in a 650 W microwave oven; then the slides were added to the solution and heated under pressurised conditions on full power for 8 min. The pressure was released and the slides were allowed to cool for 20 min before rinsing under cold water and further processing. Pepsin digestion at 37 °C was increased to 34 min.

Once pretreatment and tissue digestion were complete, all slides were examined for the adequacy of digestion [14]. Underdigested slides were digested further for a maximum of 3 min. Overdigested slides were discarded and duplicate slides digested for a reduced time (4 min less per digestion for soft tissue samples and 2 min less per digestion for bone specimens).

Dual hybridization with an AR probe (SpectrumOrange™-labelled probe, locus Xq11–13, Vysis, UK, Ltd.) and an X-chromosome alpha-satellite probe (SpectrumGreen-labelled CEP X, Vysis, UK, Ltd.) was then performed in an identical way for bone and soft tissue specimens, as described previously [14].

Following hybridization, adjacent serial haematoxylin and eosin (H&E)-stained tissue sections were examined to delineate tumour areas. The DAPI-stained FISH sections were examined on a Leica epifluorescence microscope to localize the tumour and 20 non-overlapping nuclei per section were evaluated from three different tumour sites ($\times 1000$ magnification). The signals per nucleus for the AR gene and chromosome X were counted on a cell-by-cell basis. Nuclei with either no signal for AR or X, or only one colour, were not counted [14]. A second observer scored the same tumour areas as the first observer.

AR gene copy number was calculated by totalling the number of orange signals (AR probe) counted in each specific area and dividing this figure by the number of nuclei assessed. The X-chromosome copy number was calculated similarly. These results were combined to give a mean for each probe per tumour sample. The mean AR:X ratio was then calculated. AR gene amplification was defined as a mean AR gene copy number : mean X-chromosome copy number ratio of greater than 1.5 [14].

Statistics

The inter-observer error was calculated from the absolute difference between the means for each

observer divided by the mean for both observers. If the inter-observer error was more than 20%, the specimen was reviewed again by the two observers.

Ninety-five per cent confidence intervals (CIs) for the final score for each tumour sample were also calculated. Two-tailed Fisher's exact tests were used to compare chromosome X and AR copy number and AR gene amplification between the primary and HRPC tumour specimens.

The normal ranges for AR gene copy number and X-chromosome number were derived from analysis of 14 benign prostatic hyperplasia (BPH) samples. A mean copy number ± 3 standard deviations (SD) was used to produce 99% confidence intervals for both AR gene and X chromosome copy number (0.91–1.30 and 0.97–1.23, respectively). Abnormal AR gene copy number or chromosome-X number was defined as any figure outside these ranges. The normal range for the AR gene : X chromosome ratio in the 14 BPH samples was 0.93–1.07 (1.04 \pm 3 SD).

Results

Most patients in this study (58%) presented with metastatic disease (M1); six patients had received radical surgery or radiotherapy; and four had presented with locally advanced disease (Table 1). The number

of previous treatments for prostate cancer ranged from one to six and included most curative and palliative modalities currently in use.

FISH was successful in 75% of cases (see Table 2). Three bone marrow trephines contained insufficient tumour, as 60 tumour cells could not be identified confidently from within the sample (patients 6, 7, and 21). In three autopsy samples (patients 10, 12, and 16), repeated experiments failed.

Examples of FISH analysis for samples with normal AR gene and X-chromosome copy number (Figure 1A), twice the normal X-chromosome copy number (Figure 1B), and AR gene amplification (Figure 1C) are shown.

The 18 successful HRPC samples consisted of 12 bone marrow trephines, three local recurrences, and three metastases in lymph nodes (two autopsy samples and one Trucut® biopsy). The overall AR amplification rate for these 18 HRPC cases was 9/18 (50%). The AR amplification ratios ranged from 1.66 to 5.09 (i.e. up to an average of 4.09 extra copies of AR gene per cell). Individual cell nuclei were scored as having up to 14 copies of the AR gene, but tight clusters of amplification made the copy number difficult to determine precisely in some cases. For bone metastases, the AR gene amplification rate was 5/12 (38%).

For the 13 primary tumours examined, the AR amplification rate was zero (see Table 2). The highest

Table 1. Clinical details of study patients

Patient No.	Age (years)	Stage at first presentation	Previous treatments for prostate cancer
1	69	T3	TUR, O, B, S
2	74	M1	TUR, G, F
3	76	M1	O, S & HC
4	70	T3 M1	MAB
5	78	M1	TUR, MAB, S & HC, Mitox
6	69	M1	O, CPA
7	75	pT3 N0	RP, O, F, S & HC
8	74	M1	G, Zoled, F
9	70	pT3b N0	RP, G & F, S, EcarboF, HC
10	61	M1	G & B, S & HC, EcarboF
11*	65	pT3b	RP, G & CPA, B
12	62	pT3b N1	RP, RT, G & F, P, EcarboF, S
13	72	M1	O, CPA, Z, S, B, Mitox
14	60	M1	G, Estra, S & HC
15	74	M1	TUR, G, S & HC
16	64	M1	G & B, S, Carbo
17	66	T3 M1	O, F, S & HC, EcarboF, B, Mitox
18	75	M0	TUR, G, F, Zoled
19	64	T3 N0 M0	RT, O, S & HC, EcarboF
20	73	M0	RT & G, S & HC, B
21	62	M1	G, B, P, EcarboF, S
22	64	T2 N0	RT, MAB, S & HC
23	66	M0	TUR, G
24	88	M1	G

B = bicalutamide; Carbo = carboplatin; CPA = cyproterone acetate; Estra = estramustine; EcarboF = epirubicin, carboplatin, and 5-FU; F = flutamide; 5-FU/FA = 5-fluorouracil and folinic acid; G = Goserelin; HC = hydrocortisone; MAB = maximum androgen blockade; Mitox = mitoxantrone; O = orchidectomy; P = prednisolone; S = stilboestrol; RP = radical prostatectomy; RT = radical radiotherapy; TUR = transurethral resection; Zoled = zoledronic acid.

* Co-existent chronic myeloid leukaemia.

Table 2. Results of FISH analysis of HRPC cases and primary tumours with inter-observer error rates

Sample No.	HRPC: AR/X amplification ratio (95% CI)	I-O error	Primary tumour: AR/X amplification ratio (95% CI)	I-O error	HRPC: mean copy number AR (95% CI)	I-O error	HRPC: mean copy number X-chromosome (95% CI)	I-O error	Primary tumour: mean copy number AR (95% CI)	I-O error	Primary tumour: mean copy number X-chromosome (95% CI)	I-O error
1 (TUR)	4.67 (3.75–5.70)	15%	—	—	5.04 (4.04–6.04)	2%	1.08 (1.02–1.13)	14%	—	—	—	—
2 (TUR)	1.91 (1.65–2.17)	11%	—	—	1.93 (1.64–2.21)	13%	1.01 (0.99–1.02)	2%	—	—	—	—
3 (BMT)	1.1 (1.0–1.21)	4%	—	—	1.13 (1.0–1.27)	3%	1.03 (1.0–1.05)	2%	—	—	—	—
4 (BMT)	1.11 (1.07–1.15)	6%	—	—	1.2 (1.08–1.32)	3%	1.08 (0.99–1.17)	9%	—	—	—	—
5 (BMT)	5.06 (4.39–5.8)	5%	1.04 (1.02–1.05)	5%	5.41 (4.64–6.19)	5%	1.07 (1.05–1.08)	0%	1.14 (1.08–1.21)	1%	1.1 (1.05–1.15)	3%
6 (BMT)	—	—	1.02 (1.0–1.03)	6%	—	—	—	—	1.05 (1.02–1.08)	10%	1.03 (1.02–1.05)	3%
7 (BMT)	—	—	1.02 (1.0–1.07)	3%	—	—	—	—	1.06 (1.04–1.07)	8%	1.03 (0.98–1.07)	5%
8 (BMT)	1.16 (0.88–1.42)	13%	1.24 (0.97–1.51)	4%	1.23 (0.85–1.62)	5%	1.06 (0.99–1.15)	6%	1.33 (0.88–1.77)	9%	1.07 (0.94–1.17)	5%
9 (TUR)	1.02 (0.96–1.07)	7%	1.04 (1.02–1.07)	5%	1.73 (1.25–2.2)	9%	1.7 (1.23–2.17)	16%	1.75 (1.04–2.31)	2%	1.68 (1.09–2.41)	1%
10 (PM BM)	—	—	—	—	—	—	—	—	—	—	—	—
11 (BMT)	1.27 (1.17–1.39)	1%	1.14 (1.0–1.3)	1%	1.31 (1.25–1.37)	4%	1.03 (0.98–1.07)	5%	1.29 (1.12–1.46)	6%	1.13 (1.07–1.18)	7%
12 (PM BM)	—	—	1.07 (1.03–1.11)	9%	—	—	—	—	1.10 (1.05–1.15)	3%	1.03 (1.0–1.07)	6%
13 (BMT)	1.75 (1.22–2.35)	18%	—	—	2.03 (1.52–2.55)	15%	1.16 (0.94–1.37)	1%	—	—	—	—
14 (BMT)	2.07 (1.53–2.58)	5%	—	—	2.13 (1.56–2.72)	13%	1.03 (1.02–1.06)	11%	—	—	—	—
15 (PM LN)	3.10 (2.88–3.36)	14%	1.04 (0.87–1.21)	1%	3.88 (3.21–4.56)	3%	1.25 (1.12–1.38)	11%	2.16 (1.88–2.43)	7%	2.08 (1.97–2.20)	8%
16 (PM BM)	—	—	1.07 (0.95–1.21)	5%	—	—	—	—	1.83 (1.09–2.58)	4%	1.71 (1.04–2.38)	3%
17 (LN)	1.72 (1.63–1.81)	11%	—	—	1.87 (1.71–2.19)	5%	1.09 (1.01–1.26)	6%	—	—	—	—
18 (BMT)	2.53 (1.84–3.81)	10%	1.0	0%	3.65 (3.33–3.97)	13%	1.44 (0.67–2.21)	6%	1.0	0%	1.0	0%
19 (PM LN)	1.02 (1.0–1.05)	1%	1.12 (0.98–1.27)	8%	1.11 (1.04–1.17)	2%	1.08 (1.04–1.13)	0%	1.28 (1.0–1.56)	3%	1.14 (1.03–1.26)	10%
20 (BMT)	1.0	0%	1.21 (1.14–1.27)	4%	1.0	0%	1.0	0%	1.33 (1.29–1.61)	4%	1.1 (0.96–1.32)	0%
21 (BMT)	—	—	—	—	—	—	—	—	—	—	—	—
22 (BMT)	1.42 (0.69–2.06)	6%	—	—	1.64 (0.56–2.73)	17%	1.15 (0.98–1.32)	12%	—	—	—	—
23 (BMT)	0.99 (0.95–1.05)	0%	—	—	1.02 (0.98–1.05)	3%	1.03 (1.01–1.05)	3%	—	—	—	—
24 (BMT)	1.71 (1.41–2.0)	16%	1.28 (1.17–1.39)	3%	1.76 (1.42–2.09)	20%	1.03 (1.02–1.05)	3%	1.14 (1.29–1.61)	5%	1.45 (0.96–1.32)	1%
Amplification rate 9/18 (50%)		Amplification rate 0/13 (0%)		Total abnormal AR copy number 12/18 (66%)		Total abnormal X-chromosome number 3/18 (17%)		Total abnormal AR copy number 5/13 (38%)		Total abnormal X-chromosome number 4/13 (31%)		

Normal range for chromosome \times copy number (99% CIs): 0.97–1.23. Normal range for AR copy number (99% CIs): 0.91–1.30.

AR amplification defined as mean AR copy number/mean \times copy number ratio above 1.50.

AR = androgen receptor; BMT = bone marrow trephine; HRPC = hormone-refractory prostate cancer; I-O = inter-observer; LN = lymph node; PM BM = post-mortem bone marrow; PM LN = post-mortem lymph node; TUR = transurethral resection.

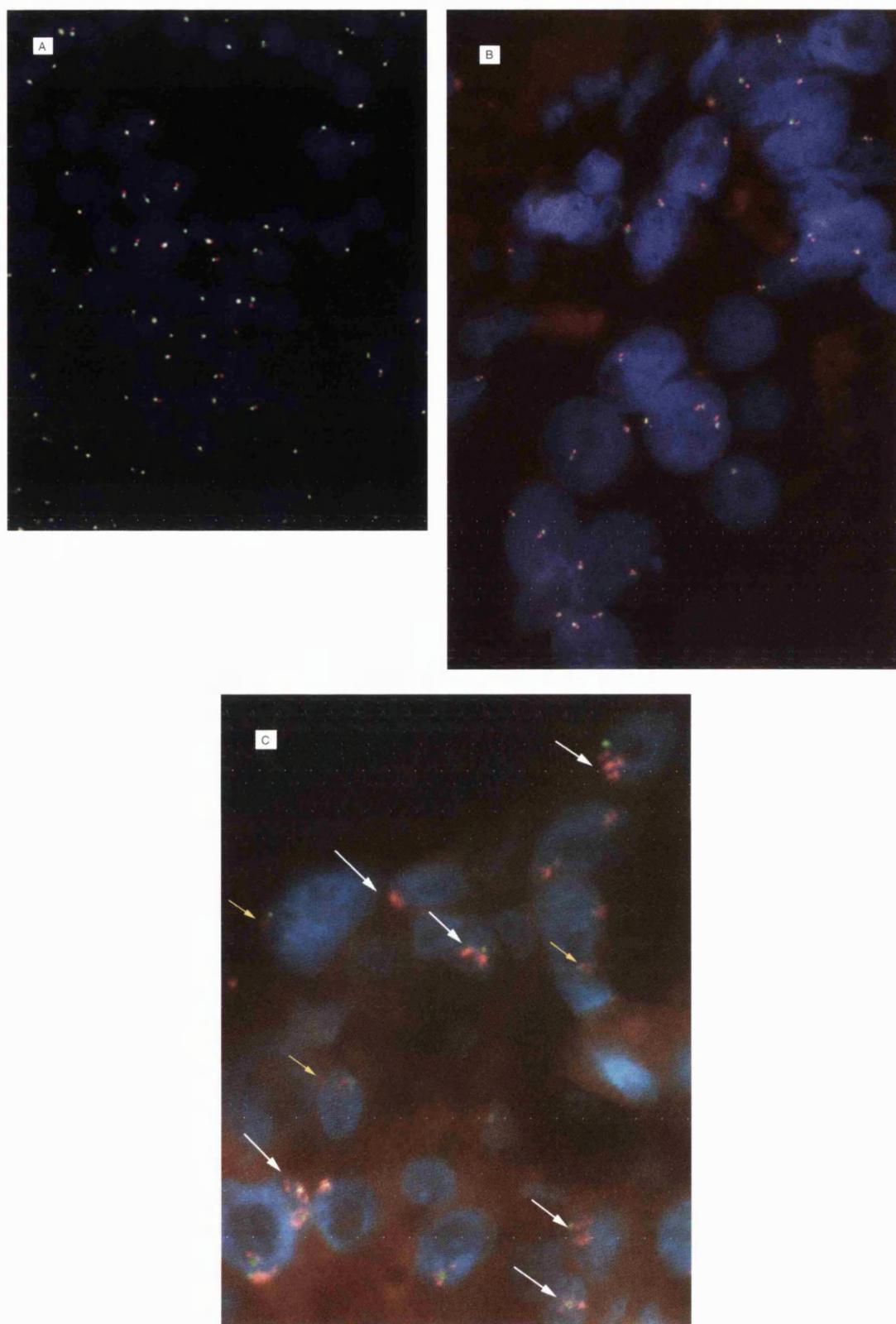


Figure 1. (A) Section of primary prostate cancer showing nuclei stained with DAPI. AR gene and X-chromosome copy number were analysed by FISH using red (AR) and green (X-chromosome) probes. Most nuclei contain one green and one red signal, indicating that each nucleus contains one X-chromosome, on which there is one AR gene. (B) FISH section showing most cells in a primary tumour specimen containing two red (AR gene) and green (X-chromosome) signals, indicating disomy. (C) FISH section from an HRPC bone marrow sample, showing multiple red AR gene signals (white arrows) associated with a single X-chromosome green signal. This indicates AR gene amplification. Note the glandular structure in the lower right corner of the picture and other areas of AR gene amplification not in the current plane of focus (yellow arrows)

AR:X ratio was 1.28. A significant difference between the AR gene amplification rates was observed between primary and HRPC cases (9/18 compared with 0/13, $p = 0.0048$, Fisher's two-tail exact, see Table 2).

Elevation of the X-chromosome copy number was observed in 4/13 primary tumour specimens (mean X-chromosome copy number 1.45, 1.68, 1.71, and 2.08) and 3/18 HRPC samples (mean X-chromosome copy number 1.25, 1.44, and 1.7). Three primary tumours (samples 9, 15, and 16) were disomic for the X-chromosome (and AR gene) copy number. In one case (sample 9), both the primary tumour and the subsequent HRPC were disomic for the AR gene (rather than the normal single gene copy number). Another of these cancers (sample 15) developed AR amplification (mean AR:X ratio increasing from 1.04 to 3.10), associated with progression to HRPC.

An elevated AR:X ratio without amplification (i.e. mean AR:X ratio of between 1.07 and 1.5) was found in 5/18 cases of HRPC and 4/13 primary tumour samples. In total, only 4/18 HRPC samples had a normal mean AR:X ratio, compared with 9/13 primary tumours ($p = 0.024$, Fisher's exact test and see Table 2).

Discussion

This is the first report of AR gene amplification in bone marrow biopsies from patients with HRPC. The molecular mechanisms underlying the development of HRPC remain unclear. AR gene amplification is found in a significant minority of cases of HRPC and by allowing efficient utilization of low levels of circulating androgens, may produce a growth advantage [6,7]. The AR signalling pathway remains functional in most cases of HRPC, both via AR signalling [12] and androgen-independent activation of AR [17].

These results show a higher frequency of AR gene amplification in HRPC than earlier studies (50% compared with approximately 30%, range 13–36%, see Table 3) [6–14]. It is apparent that AR FISH

analysis is possible on most bone metastases and levels of AR gene amplification (5/12, 38%) similar to other metastatic sites were found. We have used routine histopathological sections, whereas in contrast, most series have used isolated tumour nuclei for AR FISH [6,7,10–12]. Tissue section material was required for a number of reasons. In most other AR gene amplification studies, the material examined has been from prostatic samples, where areas of high tumour load can usually be found. Such similar levels of tumour infiltration were not always seen in the bone marrow sections that we obtained. Additionally, tumour cells within bone marrow are found against a background of rapidly dividing, non-neoplastic normal cells of variable morphology. A method that ensured accurate identification of tumour cells within bone marrow samples was therefore required. By using tissue sections, slides could be related back to immediately adjacent haematoxylin and eosin-stained sections, allowing accurate tumour cell identification under ultraviolet light.

Validation of our results against preparations of isolated tumour nuclei would have been useful but was not embarked upon because of the specificity problem. Two published comparisons using prostate tumour tissue for *in situ* hybridization, using both isolated nuclei and tissue sections from prostate cancer, suggest that the methods provide similar data [18,19]. Each has advantages and disadvantages. FISH analysis from isolated nuclei does not require special training in pathology and signal counting is easier than in tissue sections [18]. The method is also suited to large-scale studies by multiple investigators in a reasonable time frame. Tissue section analysis using FISH is slower and may suffer from nuclear truncation producing artificial monosomy.

In the small study by Alers *et al.* (eight tumours), section *in situ* hybridization was able to detect chromosomal alterations that were not seen in nuclear suspensions [19]. Chromosome copy number changes, especially gains, were better seen in nuclear suspensions. Our results show a higher frequency of AR gene

Table 3. AR gene amplification rates in HRPC and primary tumours

First author	Year	AR gene amplification rate in HRPC (No. of patients)	AR gene amplification rate in primary tumours (No. of patients)
Visakorpi	1995	30% (7/23)	0% (0/16)
Koivisto	1995	30% (3/10)	0% (0/10)
Koivisto	1997	28% (15/54)	0% (0/26)
Bubendorf	1999	23% (116)	1% (2/205)
Kaltz-Wittmer	2000	36% (22/66)	0% (0/22)
Myoshi	2000	20% (1/5)	0% (0/37)
Palmberg	2000	13% (10/77)	—
Ware	2000	—	47% (8/17)
Linja	2001	31% (4/13)	0% (0/33)
Edwards	2001	15% (3/20)	5% (1/20)

AR = androgen receptor; HRPC = hormone-refractory prostate cancer.

amplification than usually reported in the literature, suggesting that this was not a problem in our study. In addition to the two studies above that used tissue sections in comparison with isolated tumour nuclei, several other studies have used tissue sections alone for *in situ* hybridization. Bubendorf *et al.* used tissue section microarrays, in a large study that included AR gene amplification data from prostate tumour specimens and non-bony metastases [9]. Qian *et al.* and Alers *et al.* have both published studies using tissue section analysis for *in situ* hybridization [20,21]. Our findings therefore clearly show that AR gene amplification can also be reliably demonstrated in routine bone biopsy tissue sections, extending the potential of this tool in the management of HRPC.

There is no standard criterion for AR gene amplification [6,7,9–12,14]. In the present study, we have used the stringent definition of a ratio in excess of 1.5 [14]. Despite this strict definition, two cases were borderline (samples 13 and 24), in that the 95% CIs straddle the definition of AR gene amplification (mean AR:X ratio of more than 1.5). Such variability may, however, simply represent inter-observer error. For the HRPC samples, 36/54 (66%) observations had an inter-observer error of 10% or less and in 18/54 (33%) cases, the inter-observer error was between 10% and 19%.

AR copy number can also vary within individual tumours [6]. Heterogeneity of amplification was seen in one sample (mean AR:X ratios of 1.65, 1.95, and 2.53 for the three areas, respectively). For sample 24, two out of the three areas were scored as being amplified by both observers (mean AR:X ratios of 2.10 and 1.56). The remaining tumour area was scored as amplified by one observer but not the other (with the average of the two areas being just >1.5). In a third sample (sample 22), the mean AR:X ratio did not reach 1.5, but the 95% CIs did include this figure. For this specimen, one out of the three areas examined showed AR amplification (mean AR:X ratio of 2.75) and aneusomy for chromosome X number (1.33), while the other two areas were normal for AR gene and X-chromosome copy number. It remains unclear from this and other studies what level of increase in AR gene copy number may be associated with a growth advantage for tumours treated by androgen deprivation. It is also uncertain if there may be any biological difference in behaviour between those tumours with low-level amplification (e.g. 1.5–2.5) and those with higher levels. Even if only a subpopulation of cells within a HRPC develop AR gene amplification, this may be sufficient to provide a biological growth advantage and affect clinical outcome, despite not being recognized by our classification as being amplified.

The finding that AR gene amplification is rare in untreated primary tumours is in agreement with previously published reports [6–11,13,14]. In one primary tumour sample (sample 8), the upper limit of the 95% CI is above 1.5. The inter-observer error is small and in

two of the three areas examined there was no evidence of amplification. In the third area, possible low-level amplification was seen, with a mean AR:X ratio of 1.52. The overall consistency of the finding that AR gene amplification is rare in untreated prostate cancer remains one of the strongest pieces of evidence that links AR gene amplification with the progression to HRPC. Only one study has suggested AR gene amplification in untreated primary tumours (8/17 cases, 47%) [22].

Elevated copy number for chromosome X in HRPC has been previously reported in the range of 19–80% [6–8,11,14]. Our results for HRPC samples are close to the lower end of this range (17%). An increase in X-chromosome copy number without amplification has been postulated as a mechanism for the development of a proliferative advantage in tumours during anti-androgen therapy, mediated by increased expression of AR receptor levels [7,8]. A more recent study has confirmed that one additional copy of the AR gene was able to increase AR protein expression in a human HRPC xenograft model [13].

The lowest success rate in FISH analysis was from autopsy material, in agreement with Bubendorf *et al.* [9]. For bone marrow post-mortem specimens, there was a complete failure of all FISH analyses. This may be the result of acid hydrolysis of DNA in the decalcification process; similar problems can also occur in bone marrow trephine biopsies decalcified in hydrochloric acid [15]. In contrast, decalcification in 5% formic acid overnight, as used in this study, allowed successful FISH analysis [23]. The standard departmental decalcification protocol (10% formic acid for 7–10 days) for post-mortem bone samples resulted in failure, despite successful FISH in soft tissue autopsy material from the same patient.

Studies of the genetic and molecular changes in androgen-related gene expression in metastases from HRPC may be important for the management of these patients [9]. Tumours with AR gene amplification could represent a subset of HRPC that might be targeted by other treatments, for example antisense therapy [24] or geldanamycin–testosterone hybrids [25]. A recent study has suggested that AR gene amplification in local tumour recurrences at progression following androgen deprivation monotherapy is associated with a better response to second-line combined androgen blockade [12]. Growth of tumour cells exhibiting AR gene amplification may be dependent on residual androgens. This study also suggests that there may be a clinical benefit for patients in identifying AR gene amplification upon progression to HRPC [12]. The authors noted that sampling in this context should be done at metastatic sites, but obtaining such tissue was a challenge in developing predictive tests based on AR gene amplification. Bone marrow sampling was suggested as a method of achieving this. We have shown here and elsewhere [16] that bone marrow samples can be obtained routinely from HRPC patients and analysed by FISH for AR gene amplification. Studies

are now needed to relate AR gene amplification rates in bone metastases to clinical outcome, following the addition of combined androgen blockade treatment.

References

1. Mettlin C. Clinical oncology update: prostate cancer. *Eur J Cancer* 1997; **33**: 340–347.
2. McLeod DG, Crawford ED, DeAnton EP. Combined androgen blockade: the gold standard for metastatic prostate cancer. *Eur Urol* 1997; **32**(Suppl 3): 70–77.
3. Keane P, Waxman J, Sikora K. Urological system. In *Treatment of Cancer* (3rd edn), Price P, Sikora K (eds). Chapman & Hall Medical: London, 1995; 557–585.
4. Logothetis CJ, Vogelzang NJ. Current treatment and investigative approaches to the management of hormone-refractory prostate cancer. In *Genitourinary Oncology* (2nd edn), Vogelzang NJ, Scardino PT, Shilphey WU, Coffey DS (eds). Lippincott, Williams & Wilkins: Philadelphia, 2000; 862–869.
5. Culig Z, Hobisch A, Bartsch G, Klocker H. Androgen receptor — an update of mechanisms of action in prostate cancer. *Urol Res* 2000; **28**: 211–219.
6. Visakorpi T, Hyytinen E, Koivisto P, et al. *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. *Nature Genet* 1995; **9**: 401–406.
7. Koivisto P, Kononen J, Palmberg C, et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res* 1997; **57**: 314–319.
8. Koivisto P, Hyytinen E, Palmberg C, et al. Analysis of genetic changes underlying local recurrence of prostate carcinoma during androgen deprivation therapy. *Am J Pathol* 1995; **147**: 1608–1614.
9. Bubendorf L, Kononen J, Koivisto P, et al. Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence *in situ* hybridization on tissue microarrays. *Cancer Res* 1999; **59**: 803–806.
10. Kaltz-Wittmer C, Klenk UAG, Aust DE, et al. FISH analysis of gene aberrations (MYC, CCND1, ERBB2, RB, and AR) in advanced prostate cancer before and after androgen deprivation therapy. *Lab Invest* 2000; **80**: 1455–1464.
11. Miyoshi Y, Uemura H, Fujinami K, et al. Fluorescence *in situ* hybridization evaluation of c-myc and androgen receptor gene amplification and chromosome anomalies in prostate cancer in Japanese patients. *Prostate* 2000; **43**: 225–232.
12. Palmberg C, Koivisto P, Kakkola L, Tammela TLJ, Kallioniemi OP, Visakorpi T. Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. *J Urol* 2000; **164**: 1992–1995.
13. Linja MJ, Savinainen KJ, Saramaki OR, Tammela T, Vessela RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res* 2001; **61**: 3550–3555.
14. Edwards J, Krishna NS, Mukherjee R, Watters AD, Underwood MA, Bartlett JMS. Amplification of the androgen receptor may not explain development of androgen independent prostate cancer. *Br J Urol* 2001; **88**: 633–637.
15. Alers J, Krijtenberg P-J, Vissers K, van Dekken H. Effect of bone decalcification procedures on DNA *in situ* hybridisation and comparative genomic hybridisation: EDTA is highly preferable to a routinely used acid decalcifier. *J Histochem Cytochem* 1999; **47**: 303–309.
16. Brown RSD, Dogan A, Ell PJ, Payne HA, Masters JRW, Harland SJ. The comparative value of bone marrow aspirae and trephine for obtaining bone scan-targeted metastases from hormone refractory prostate cancer. *Prostate Cancer Prostate Dis* 2002; **5**: 144–151.
17. Sadar M. Androgen independent induction of prostate specific antigen gene expression via cross talk between the androgen receptor and protein kinase A signal transduction pathways. *J Biol Chem* 1999; **274**: 7777–7783.
18. Qian J, Bostwick DG, Takahashi S, et al. Comparison of fluorescence *in situ* hybridization analysis of isolated nuclei and routine histological sections from paraffin-embedded prostatic adenocarcinoma specimens. *Am J Pathol* 1996; **149**: 1193–1199.
19. Alers JC, Krijtenberg P-J, Vissers KJ, Krishnadas SK, Bosman FT, van Dekken H. Interphase *in situ* hybridization to disaggregated and intact tissue specimens of prostatic adenocarcinomas. *Histochem Cell Biol* 1995; **104**: 479–486.
20. Alers J, Krijtenberg P-J, Vissers K, Bosman FT, van der Kwast TH, van Dekken H. Interphase cytogenetics of prostatic adenocarcinoma and precursor lesions: analysis of 25 radical prostatectomies and 17 adjacent prostatic interepithelial neoplasias. *Genes Chromosomes Cancer* 1995; **12**: 241–250.
21. Qian J, Bostwick DG, Takahashi S, et al. Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence *in situ* hybridization. *Cancer Res* 1995; **55**: 5408–5414.
22. Ware MA, Abel PD, Madaan S, Stamp GWH, Lalani E. Evidence for amplification of the androgen receptor gene in prostate cancer prior to androgen ablation therapy. *Proc AACR* 2000; **41**: 132.
23. Brown RSD, Edwards J, Bartlett JW, Jones C, Dogan A. Routine acid decalcification of bone marrow samples can preserve DNA for FISH and CGH studies in metastatic prostate cancer. *J Histochem Cytochem* 2002; **50**: 113–115.
24. Eder IE, Culig Z, Ramoner R, et al. Inhibition of LNCaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther* 2000; **7**: 997–1007.
25. Kuduk SD, Harris TC, Zheng FF, et al. Synthesis and evaluation of geldanamycin–testosterone hybrids. *Bioorg Med Chem Lett* 2000; **10**: 1303–1306.