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ANDROGEN RECEPTOR MUTATION IN BREAST CANCER

Youssef A. Elhaji
Department of Biology
McGill University, Montreal

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For my parents, my family and my Libya.

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ABSTRACT

Normal breast growth and development depends on functional androgen:estrogen (A:E) balance. Androgen actions are mediated by the androgen receptor (AR), a DNA-binding, transcriptional-regulatory protein. Decreased AR transactivational activity lowers A:E balance and may result in functional hyperestrogenicity: this could promote the pathogenesis of breast cancer (BC). The present study is the first to seek AR mutations in female BC. The length of the polymorphic CAG-repeat in exon 1 of the *AR* correlates inversely with the transactivational activity of the AR. Using 10% polyacrylamide gels, I found a significant ($p < 0.0001$) shift to greater CAG-repeat lengths in BC samples. This suggests a role for ARs with long polyglutamine tracts in the initiation and/or progression of BC. Exons 2-8 of the *AR* in 81 fresh frozen BC tumor tissues were screened for mutations using SSCP analysis. I did not detect any mutations in these exons.

Le dévelopment et la croissance normal des seins dépendent du rapport d'androgène à l'oestrogène (A:O). L'action des androgènes est médiée via le récepteur d'androgènes, une protéine qui lie l'ADN et transactive un grand nombre de gènes. Une baisse dans la transactivation du récepteur androgène (AR) contribue à une baisse dans le rapport A:O et ainsi une augmentation de l'action des oestrogènes. Cette augmentation pourrait promouvoir la pathogenèse du cancer du sein. Le but de ma recherche était d'identifier des potentiels mutations dans l'AR dans le cancer du sein chez les femmes. Mes études ont démontré qu'il y avait une corrélation inverse entre la longueur de l'insère polyglutamine (polyQ) dans le premier exon de l'AR et la transactivation de l'AR. En utilisant des gels de polyacrylamide de 10%, j'ai trouvé une augmentation significative ($p< 0.0001$) de l'expansion de l'insert poly-Q dans les échantillons des femmes atteintes du cancer du sein. Ses résultats suggèrent un role pour l'AR avec une expansion de l'insère poly-Q dans l'initiation ou la progression du cancer du sein. Les exons 2 à 8 de l'AR dans 81 échantillons ont été analysé pour des mutations en utilisant SSCP. Je n'ai pas trouvé de mutations dans ces exons.

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A	Androgen
ABD	Androgen-binding domain
AI	Androgen insensitivity
AIS	Androgen insensitivity syndrome
Ala	Alanine
AR	Androgen receptor
ARE	Androgen-response element
Arg	Arginine
BC	Breast cancer
Bcl-2	B-cell leukaemia oncogene-2
bp	Base pair
BRCA1, 2	Breast cancer susceptibility genes 1 and 2
C-terminal	Carboxyl-terminal
CAIS	Complete androgen insensitivity syndrome
CaP	Prostate cancer
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
DRPLA	Dentatorubral-pallidoluysian atrophy
E	Estrogen
EcR	Ecdysone receptor
EGF	Epidermal growth factor
ER	Estrogen receptor
ERE	Estrogen-response element
Gln	Glutamine
GR	Glucocorticoid receptor
GRE	Glucocorticoid-response element
hAR	Human androgen receptor
HD	Huntington's disease
HRE	Hormone-response element
IGF-1	Insulin-like growth factor-1

kb	Kilobase pair
kDa	Kilodalton
LBD	Ligand-binding domain
LH	Luteinizing hormone
LNCaP	Cell line derived from human lymph node carcinoma of the prostate
Lys	Lysine
MAIS	Mild androgen insensitivity syndrome
Met	Methionine
MR	Mineralocorticoid receptor
N-terminal	Amino-terminal
nHRE	Negative hormone-response element
NLS	Nuclear localization signal
PAIS	Partial androgen insensitivity syndrome
PR	Progesterone receptor
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SBMA	Spinal and bulbar muscular atrophy
SCA1-3,6 & 7	Spinocerebellar ataxia types 1-3,6, and 7
SHBG	Sex-hormone binding globulin
SSCP	Single-strand conformation polymorphism
T	Testosterone
TGF-α	Tumor growth factor-α
Thr	Threonine
TIS I & II	Transcription initiation sites I and II
TR	Thyroid receptor
UTR	Untranslated region
Val	Valine
VDR	Vitamin D receptor
Zn	Zinc

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If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

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Breast cancer (BC) is likely to be the end result of an accumulation of multiple aberrations in several genes that are critical for cell proliferation, differentiation and apoptosis. The combinations of these aberrations may vary from one BC to another. However, an estrogenic environment in breast cells is apparently essential in nearly all of BCs. The female:male ratio for BC is 100:1. Almost certainly, this reflects the higher levels of estrogen in women compared to that in men. In addition, BC is observed only after menarche, and women with higher lifetime exposures to estrogen are at higher risk of BC (Pike et al., 1981; Feinleib, 1968). Estrogen promotes and maintains breast tumor growth and inhibits apoptosis, and androgen exerts opposite (protective) effect. The protective effect of androgen has been repeatedly demonstrated in BC cell lines (Szelei et al., 1997; Couture et al., 1993; Boccuzzi et al., 1993; Hackenberg et al., 1991). Androgen actions are mediated by the androgen receptor (AR). A defective or deficient AR in a breast cell, due to mutations at the *AR* locus, may lower the androgen:estrogen (A:E) balance in that cell and, thereby, generate functional hyperestrogenicity that promotes the pathogenesis of BC. However, the role of the AR in BC is not well understood, and AR mutations in female BC have never been documented.

To investigate this role, I addressed the question of whether AR mutations can be incriminated in the pathogenesis of female BC among females who may be constitutionally predisposed or environmentally exposed.

The nuclear receptor superfamily, comprising more than 150 different proteins, is the largest known family of transcription factors in eukaryotes (Tsai and O'Malley, 1994; Mangelsdorf and Evans, 1995). Nuclear receptors are ligand-activated, DNA-binding, transcription-regulating proteins (Evans, 1988; Green and Chambon, 1988; Beato, 1989). Nuclear receptors, unlike membrane-bound receptors, link extracellular signals directly to transcriptional responses to perform extremely diverse functions in homeostasis, reproduction, development, differentiation and apoptosis. As defined by primary structure homology, the nuclear receptor superfamily includes receptors for steroids: estrogen (ER), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR), and nonsteroids: thyroid (TR), retinoids (RAR and RXR), and ecdysone (EcR). Also, a variety of isoforms of TR, RAR, RXR, ER, PR, and EcR have been identified (Tsai and O'Malley, 1994). In addition, cloning by various means has identified a large number of proteins that by homology belong to the nuclear receptor superfamily. Since, no regulatory ligand has been identified for these proteins, they have been termed the orphan receptors (Mangelsdorf et al., 1995; Laudet et al., 1992). The receptor function of the orphan receptors is hypothetical: many of them are likely to be simply transcription factors that happen to display homology with the nuclear receptor superfamily (Mangelsdorf and Evans, 1995). Besides analogy of structure, there are several other candidates for membership in the nuclear receptor superfamily based solely on their mechanism of action. These are proteins known to bind lipophilic molecules such as dioxin and vitamins E and K (Mester et al., 1995).

The Modular Structure-Function Organization of The Nuclear Receptors

Though nuclear receptors vary in length from 427 amino acids for VDR to 984 amino acid for MR, they are composed of comparable domains (Fig. 1) (reviewed in Tsai and O'Malley, 1994; Mester et al., 1995). The N-terminal domain contains a transactivational modulatory function and may be important also for the specificity of

185 315 553
1 66 595 ER

567 687
1 66 933 PR (B)
(A) 768

421 532
1 66 777 GR

603 738
1 66 984 MR

558 669
1 66 918 AR

24 196
1 66 427 VitD3

88 200 420
1 66 462 RAR α

81 193 413
1 66 448 RAR β

53 189 371
1 68 490 TR α

102 238 420
1 68 456 TR β

OH
HO
(R) R
O
OH
OH
HO
COOH
I I
HO O CH_2 - C - NH_2
H
I
COOH

Fig. 1. Members of the human nuclear receptor superfamily and their ligands (Jensen, 1991). The top diagram shows the modular structure of nuclear receptors. Boxes indicate highly conserved domains. The boundary of each domain is given as the number of amino acids from the amino terminus. Numbers of amino acids in the DNA-binding domains are indicated in the C boxes.

receptor isoforms. This domain is variable in sequence and length, and accounts for most of the variability in nuclear receptor sizes. The central domain, the DNA-binding domain (DBD), is partly responsible for dimerization and DNA recognition and binding. The DBD has the highest degree of conservation among nuclear receptors. It contains two zinc (Zn) fingers each of which contains a Zinc ion coordinated by four cysteine residues. Each Zn finger is flanked C-terminally by an α-helix, that starts between the third and fourth cysteines, followed by an extended region. In addition to DNA binding and dimerization, the DBD may contribute to transcriptional regulation. The C-terminal ligand-binding domain (LBD) contains sequences responsible for ligand binding and specificity. The entire length of the LBD is required for normal ligand-binding, a single amino acid change can abolish the binding or alter the specificity of a receptor for a given ligand. In addition to ligand binding, this domain contains regions for heat-shock proteins association, dimerization, and nuclear localization. The portion between the DBD and the LBD serves as a hinge region, which may allow the protein to bend or alter its conformation, and often contains a nuclear localization domain and/or transactivation domain. However, the modular structure of the different members of the nuclear receptor superfamily is not always exactly the same. Also, the division of labor is incomplete and several functions require interactions between individual domains. In the androgen receptor (AR) for instance, interactions between the N- and C-terminal domains of the unliganded receptor repress the transactivational activity of the N-terminal domain (Rundlett et al., 1990), and inhibit the nuclear targeting signal (Zhou et al., 1994). In the liganded AR, these interactions enhance the retention of the androgen (Zhou et al., 1995). Also, AR fragments containing the DBD with the N- or C-terminal domains form heterodimers in association with specific DNA binding (Wong et al., 1993).

Another feature of the nuclear receptor superfamily is the correspondence between the exon/intron structure of the gene and the functional domains of its product. The transactivational domain and each of the two Zn fingers of the DBD are encoded by separate exons.

Mechanism of Action of the Nuclear Receptors

Ligand-Receptor Interaction

A two-step mechanism has been recognized for the action of nuclear receptors. The native (untransformed) receptors are located in both the cytoplasm and the nucleus. Upon binding their respective ligand, nuclear receptors undergo an activation (transformation) step, in which conformational changes, dissociation of hsp90 and other proteins, dimerization and several phosphorylations occur. The second step is the intracellular and/or intranuclear translocation of the ligand-receptor complexes to bind and interact with their target genes, leading to the activation or repression of transcription of those genes (Brann et al., 1995; Jensen, 1991; Gronemeyer, 1992; Beato, 1989). However, the two-step mechanism applies only to the recognized functional receptors and, as mentioned previously, many of the nuclear receptors are simply ligand-independent transcription factors.

In addition to their classical ligand-receptor complex action, receptors and hormones can act independently. Nuclear receptors have been shown to be activated by agents that stimulate intracellular phosphorylation pathways and by growth factors such as IGF-1, EGF, and TGF-α. Some of these events involve a 'cross-talk' between membrane and nuclear receptors (reviewed in Tsai, 1994). A recent study suggested that the nuclear receptors may shuttle continuously between the nucleus and the cytoplasm (Guiochon-Mantel et al. 1996), and that the same NLSs are involved in both inward and outward movements of the receptors. This shuttle suggests that nuclear receptor may undergo some modifications and exert some biological functions in the cytoplasm. On the other hand, extensive evidence suggests that hormones, and their metabolites, may exert receptor-independent effects. These effects occur so rapidly (seconds to minutes) that they preclude the classical mechanism. In addition, they occur even if the hormone is not permitted to enter the cell, thus they are mediated at the plasma membrane level (Brann et al., 1995 and refs. therein).

Protein-DNA Interactions: Hormone Response Elements (HREs) and DNA-Binding Domain (DBD)

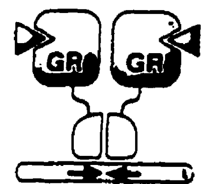
At their target genes, the active receptors bind specific DNA sequences called hormone response elements (*HREs*) or negative *HREs (nHREs)* to activate or repress transcription initiation respectively. *HREs* and *nHREs* function in a position- and orientation-independent fashion, thus *HREs* act like enhancers and *nHREs* like silencers (Pinsky et al., 1994; Evans 1988). The *HREs* are composed of two 6-base pair half-sites that are organized as direct or inverted (palindromic) repeats separated by spacers of variable number of nucleotides. Nuclear receptors bind mainly as dimers, and each monomer interacts with a half-site of the response elements.

Based on dimerization and DNA-binding properties, the nuclear receptor superfamily can be divided into four classes (fig. 2): steroid receptors bind as homodimers to inverted repeats separated by three nucleotides; receptors that heterodimerize with RXR and bind to direct repeats; receptors that bind to direct repeats as homodimers; and receptors that bind to extended core sites as monomers (Mangelsdorf et al., 1995).

The specific functions of nuclear receptors depend upon the genes that they regulate. Part of the recognition of target genes is due to the specificity of the *HREs* with which receptors interact. Most nuclear receptors, including ER, TR, RAR, and VDR, as well as most orphan receptors, recognize the estrogen response element (ERE) consensus half-site TGACCT. The specificity within this group is mainly due to the difference in organization and spacing between the half-sites of the *HREs*. Another group, including GR, PR, MR, and AR, recognize the glucocorticoid response element (GRE) consensus TGTTCT. However, no clear differences in the DNA-binding specificity have been detected within this group (reviewed in Zilliacus et al., 1995; Mangelsdorf and Evans, 1995).

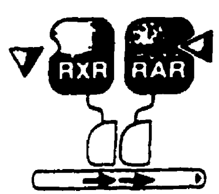
As suggested by the conservation among the *HREs*, the amino acids responsible for DNA binding are also conserved. The N-terminal α-helix of each monomer contacts and

Steroid Receptors



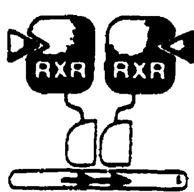
GR	*glucocorticoid*
MR	*mineralcorticoid*
PR	*progesterone*
AR	*androgen*
ER	*estrogen*

RXR Heterodimers



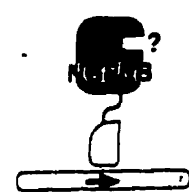
$TR_{\alpha,\beta}$	*thyroid hormone*
$RAR_{\alpha,\beta,\gamma}$	*all-trans RA*
VDR	*$1,25\text{-}(OH)_2\text{-}VD_3$*
$PPAR_{\alpha,\beta,\gamma}$	*eicosinoids*
EcR	*ecdysone*

Dimeric Orphan Receptors



RXR (usp)	*9-cis RA*
COUP/ARP	?
HNF-4	?

Monomeric Orphan Receptors



NGFI-B (CEB-1')	?
ELP / SF-1 (Ftz-F1)	?

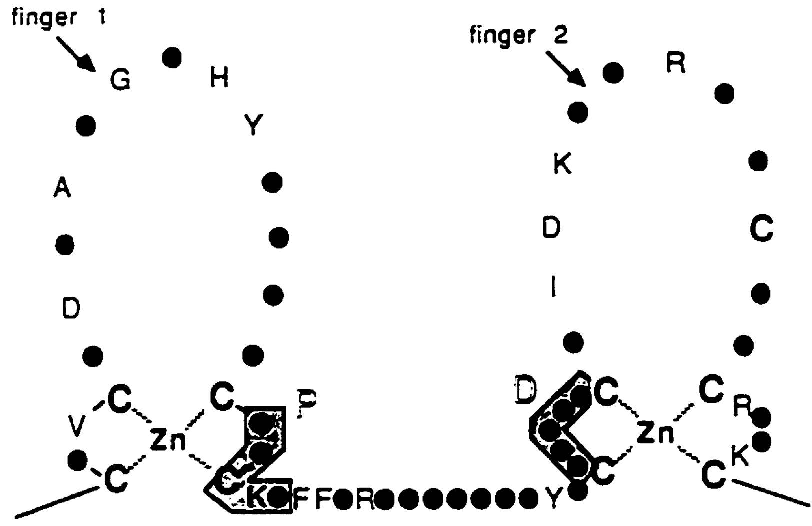
Fig. 2. Classes of nuclear receptor superfamily (Mangelsdorf et al., 1995). The nuclear receptors have been grouped into four classes according to their ligand-binding, DNA-binding, and dimerization properties. Steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors.

fits into a major groove of the DNA helix, while the C-terminal Zn fingers are responsible mainly for dimerization (fig. 3). The N-terminal (proximal) and C-terminal (distal) Zn fingers contain short elements of amino acids termed the P-box and the D-box respectively. Three amino acids in the P-box are essential for the specificity of DNA-binding. In addition to dimerization, the D-box is important for recognizing the spacing between the two half-sites (Tsai et al., 1994; Mester et al., 1995).

Upon DNA binding nuclear receptors interact, either directly or through intermediate factors, with the transcriptional machinery to enhance or repress transcription. They enhance transcription by the formation of stable preinitiation complexes at their target promoters. Negative *HREs* appear to force the receptor into a conformation that exposes peptide domains that silence transcription. Also, a subgroup of smaller nuclear receptors, including TR, can bind to DNA in the absence of ligand and silence basal promoter activity (Tsai et al., 1994).

Factors influencing receptor action

Several factors influence the interactions of nuclear receptors with their target *HREs* to regulate gene transcription. *HREs* are usually found in multiple copies and in close conjunction with other *cis*-acting elements. This allows protein-protein interactions among receptors and with other transcription factors and cofactors (Horwitz et al., 1996). However, some of these proteins act only as 'bridging' proteins and do not bind directly to DNA. Chromatin structure is another factor which, depending on the active/inactive state of the gene, alters transcription factors - DNA interaction. Also, other factors such as the local availability of transcription factors and their binding sites, as well as phosphorylation play a role in the expression of target genes (Horwitz et al., 1996; Tsai et al., 1994).


finger 1
finger 2
Zn
Zn

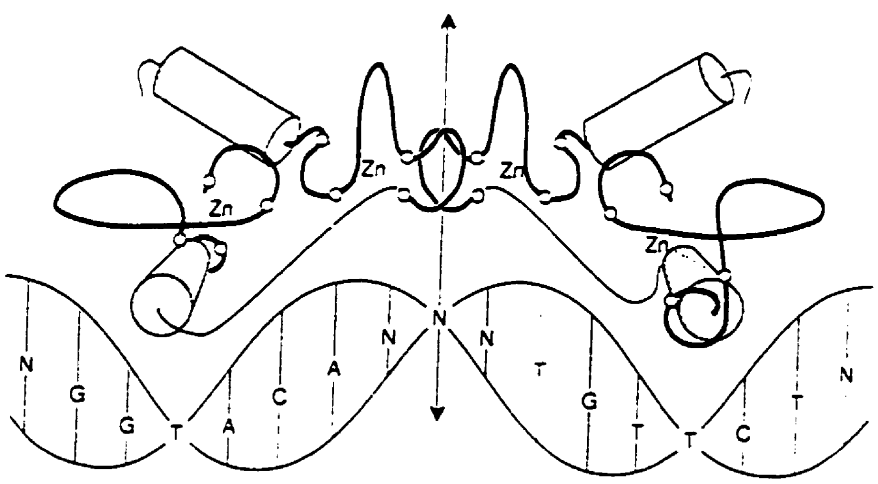

Zn
Zn
Zn
Zn

Fig. 3. The DNA-binding domain of the nuclear receptor superfamily. (top) The two Zn fingers, the P- and D- boxes and the conserved residues of the DNA-binding domain are shown (Chin, 1991). (Bottom) A model of the dimeric DNA-binding domain of the GR showing the arrangement of α-helices and Zn fingers in relation to a GRE (Muller and Renkawitz, 1991).

The androgen receptor (AR) is an androgen-modulated nuclear receptor that mediates the diverse actions of androgen throughout the body. It is predominantly or exclusively nuclear, and expressed in different tissues and cell lines (Pinsky et al., 1994; Ruizeveld de Winter et al., 1991). The AR regulates, positively or negatively, the expression of androgen-responsive genes at both transcriptional and posttranscriptional levels (Pinsky et al., 1992). It is encoded by the *AR* gene which is located in the q11-q12 region of the X chromosome (Brown et al., 1989). The entire gene is 80-90 kb long, but most of it is intronic or in flanking untranscribed regions (Kuiper et al., 1989). The coding region, consisting of 8 exons, is only 2730-57 bp (Fig. 4). The promoter region of the *AR* does not contain a typical TATA or CCAAT box, but does contain GC-rich sequences, including a binding site for the transcription factor SP-1, a homopurine-rich region, and a cAMP response element (Tilly et al., 1990; Song et al., 1993; Faber et al., 1991; Mizokami et al., 1994). The promoter contains two transcriptional initiation sites. The principal site, TIS I, is located ~1100 bp 5′ of the translation-initiating methionine and is used in all tissues and cell lines studied. A minor site, TIS II, the role of which is unknown, is located 11 nucleotides downstream of TIS I (Tilly et al., 1990). There are two AR-mRNA species, a major of ~10 kb and a less abundant of ~7kb, differing in length of the 3′ UTR due to alternative splicing (Wolf et al., 1993; Faber et al., 1991). The translated product is composed of 910-919 amino acids calculated to be ~98-99 kDa (Brinkmann et al., 1989; Lubahn et al., 1988).

The *AR* retains the common intron/exon organization and the modular structure/function organization of the nuclear receptors. Exon 1 codes for the modulatory region which, in contrast to other steroid receptors, contains two polymorphic polymeric-repeats. The first repeat, starting at codon 58, is a polyglutamine repeat ranging from 11-33 repeats encoded by $(CAG)_n$. The second repeat is a polyglycine repeat that ranges from 12-29 repeats starting at codon 449. It is these polymorphic repeats that account for the length variation among the human androgen receptor (hAR) cDNA clones (Quigley et al., 1995; Pinsky et al., 1994). The

DNA

Introns (kb)

>24 >15 26 5.6 4.8 0.8 0.7

Exon1 2 3 4 5 6 7 8

1.1 kb UTR

6.8 kb UTR

mRNA

5' UTR

Exon 1 2 3 4 5 6 7 8

3' UTR

Exons (bp)

1586 152 117 288 145 131 158 153

Protein

N Q Modulatory G DBD ABD C

11-33 12-29

Fig. 4. The intron/exon structure of the human androgen receptor (*AR*) gene and the modular structure/function of its product. The N-terminal transactivation domain is encoded by exon 1, the central DNA-binding domain is encoded by exons 2 and 3, and the C-terminal androgen-binding domain is encoded by exons 4-8.

DBD of the AR , encoded by exons 2 and 3, contains 66 amino acids. It shares ~80% sequence homology with other members of the AR subfamily, including MR, PR and GR. Moreover, the three amino acids, in the P-box in the first Zn finger, responsible for the recognition of the *HREs* are identical among the subfamily members, thereby binding the same HRE-consensus (Zilliacus et al., 1995; Mangelsdorf and Evan, 1995). However, the AR also binds to specific androgen response elements (AREs) that contain, besides the *HREs*, recognition sites for other transcription factors (Quigley et al., 1995). The hinge region of the AR, encoded by the C-terminal portion of exon 3 and the N-terminal portion of exon 4, contains a bipartite NLS that mediates the translocation of the AR (Zhou et al., 1994). This region may also contribute to the homodimerization of the AR (Wong et al., 1993). The ABD of the AR is ~250 amino acids encoded by the C-terminal portion of exon 4 and exons 5-8. Deletions in this domain as well as a truncation of the last 12 C-terminal residues abolish hormone binding. This implies that the entire length of the ABD is required for normal binding to occur. However. large and complete deletions of this domain result in constitutively active AR (Rundlett et al., 1990), indicating that the unliganded ABD has a repressive effect on the AR transcriptional activity. The consequence of androgen binding, accompanied by conformational changes in the AR, is derepressive.

Testosterone and Dihydrotestosterone

As do most nuclear receptors, the AR follows the two-step mechanism of action (activation by hormone-binding then translocation and DNA-binding). The AR is bound and activated mainly by testosterone (T) and 5α-dihydrotestosterone (DHT). Adrenal androgens and other steroids such as progesterone also bind the AR, but with relatively low affinity. Although T and DHT exert their actions via the same receptor, they play different roles during morphogenesis and at puberty. T is responsible for the development of the male internal reproductive organs such as epididymis, vas deferens and seminal vesicles whereas DHT promotes the virilization and growth of the prostate and the external genitalia. Normal androgen responsiveness depends on the presence of the correct androgen in target cells. The conversion of T to DHT requires the 5α-

reductase enzyme and occurs within the targets of DHT action. Therefore, the division of labor between the two is regulated, at least in part, by the availability of 5α-reductase in the target cell. The mechanism(s) by which the two ligands exert different actions is not fully understood. However, one possibility is their different effects on the receptor stability. The AR is rapidly degraded when unliganded and requires prolonged occupancy with androgen for its stabilization and function. DHT has higher binding affinity for and slower dissociation (~3 times) from the AR compared to its precursor T (Zhou et al., 1995), thereby is more effective. Yet, at higher concentrations, where receptors are constantly liganded, T and DHT exert similar stabilization effects. Another possible mechanism is the induction of different conformational changes in the activated receptor, which would permit interactions with different transcription factors that result in the activation of different genes.

Regulation of The AR

Autoregulation of the AR, both at the transcriptional and post-transcriptional levels, is a complex, age- and tissue-dependent process. Among various cell types, androgens have contrasting effects on both mRNA and protein levels of the AR. Overall, androgen down-regulates the AR mRNA levels, by reducing transcription and/or half-life, and up-regulates the AR protein, by increasing its stability (Quigley et al., 1995 and refs. therein). Also, functional inactivation of the AR protein after prolonged exposures to androgens has been shown in prostate and breast cancer cell lines (Wolf et al., 1993). Recently, Dai and Burnstein (1996) identified two androgen response elements in the AR coding region involved in cell line-specific up-regulation of AR mRNA. Other hormones and growth factors such as follicle-stimulating hormone and epidermal growth factor have been found to regulate AR in different cell types (Quigley et al., 1995 and refs. therein).

Androgen Insensitivity Syndrome (AIS)

A functional AR is required to mediate the diverse actions of androgens that are essential for male and female sexual development. Mutations at the *AR* locus, depending on their kind and position, result in various degrees and patterns of androgen insensitivity in 46,XY individuals (Quigley et al., 1995; Brinkmann et al., 1995; Pinsky et al., 1994). Since the *AR* is encoded by a single X chromosome in males, any mutation that causes AR deficiency results in a degree of androgen insensitivity (one mutation ⇒ one phenotype). Furthermore, these mutations do not reduce viability even if they are reproductive (genetic) lethals. It is therefore not surprising that androgen insensitivity is more common than any other form of steroid resistance, and that mutations in the AR are reported more than in any other transcription factor (Semenza, 1994). AIS ranges from complete (CAIS) to mild (MAIS), with a wide range of partial insensitivity (PAIS) in-between. Phenotypically, the spectrum ranges from an XY female to nearly normal male: CAIS, female external genitalia with absence of pubic or axillary sexual hair after puberty; PAIS, a wide spectrum from nearly male to nearly female external genitalia; MAIS, normal male external genitalia or small penis and scrotum with or without gynecomastia and/or oligo/azoospermia (Quigley et al., 1995; Brinkmann et al., 1995; Pinsky et al., 1994). The frequency of CAIS is usually quoted as ~1:50,000. However, this frequency is an underestimate and based only on the finding of testes in inguinal hernias of infant girls (Pinsky et al. 1994). PAIS is difficult to diagnose and MAIS may blend with the lower limits of normal males, thus, it is difficult to estimate their frequencies. Nevertheless, they are at least as frequent as CAIS.

More than 300 different mutations have been reported in the *AR* gene so far, most of which cause AIS. These mutations include complete or partial *AR* deletion, 1-4 bp deletions/insertions, single base mutations, and CAG-repeat expansions (Gottlieb et al., 1997). The vast majority of these mutations are within the DNA- and steroid-binding domains. While CAIS is associated with deletions, and single base mutations that disturb splicing, cause amino acid substitutions, or introduce premature termination codon, PAIS is predominantly associated with amino acid substitutions.

Spinal and Bulbar Muscular Atrophy (SBMA)

The polymorphic CAG repeat in exon 1 of the AR ranges from 11-33 repeats (Irvine et al., 1995; Stanford et al., 1997; Giovannucci et al., 1997). Expansions in this repeat to ≥ 40 repeats causes a recessive, adult-onset motor neuropathy, spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease (Kennedy et al., 1968; La Spada et al., 1991). So far, CAG-repeat expansions are associated with at least seven more late-onset neurodegenerative diseases that involve progressive degeneration of selective neuron populations; Huntington's disease (HD), Dentatorubral-pallidoluysian atrophy (DRPLA), and Spinocerebellar ataxia types 1-3,6 and 7 (SCA1-3,6 & 7) (Perutz, 1996; Ashley et al., 1995; Zhuchenko et al., 1997). Expanded CAG alleles exhibit meiotic instability (greater in males and more often towards further expansion) with succeeding generations, providing the molecular basis for anticipation (younger age-of-onset and increasing disease severity in successive generations) (La Spada et al., 1992; Biancalana et al., 1992). The expanded CAG repeats in the causative genes are located in the coding region and are translated to polyglutamine tracts (Housman, 1995). The mechanism(s) by which expanded polyglutamine tracts cause neuronal degeneration is not known. Furthermore, except for the AR, none of the causative genes has a known function. Nonetheless, the resemblance among these diseases indicates a similar mechanism of pathogenesis.

SBMA is characterized by atrophy of spinal and bulbar muscle, and degeneration of spinal motor neurons. In addition, affected males display signs of late-onset mild androgen insensitivity, including gynecomastia, reduced fertility, and testicular atrophy. However, these males have a normal masculinization and often are fertile in early adulthood (La Spada et al., 1992). Among all the known AR mutations, including complete deletions, only CAG-expansions cause SBMA. This indicates that, along with the loss-of-function causing MAIS, the expanded polyglutamine tract must endow the AR with a gain of function that causes SBMA. The gain of function could be simply an exaggeration of an otherwise normal AR function or the acquisition of an abnormal function.

The gain-of-function is likely to result from abnormal protein-protein interactions involving the expanded polyglutamine. Noncovalent protein interactions have been suggested, where glutamine repeats within proteins form pleated sheets of β strands held together by hydrogen bonds between their amides (Perutz et al., 1996). Another proposed mechanism involves transglutaminase activity where accumulation of ε–γ glutamyl lysine isopeptides may cause neurotoxicity (Green, 1993). Several other mechanisms that are specific to individual diseases and to specific cell types have been suggested (Scherzinger et al., 1997; Davies et al., 1997; Ross, 1995).

The length of the polymorphic CAG repeat in the normal population ranges from 11-33 (Irvine et al., 1995; Stanford et al 1997; Giovannucci et al., 1997), and correlates inversely with the transactivational activity of the AR (Tut et al., 1997; Kazemi-Esfarjani et al., 1995; Chamberlain et al., 1994; Mhatre et al., 1993). Based on a previous report from our laboratory (Kazemi-Esfarjani et al., 1995), Giovannucci et al. estimated that six CAG repeats cause a 12% difference in the AR transcriptional activation (Giovannucci et al., 1997). AR with an appreciable decrease in the transcriptional activity due to an expanded polyglutamine tract, though sufficient in early adulthood, could lead to a late-onset MAIS. The reduction of AR activity in SBMA patients has been demonstrated in genital skin fibroblasts established from SBMA patients using a recombinant adenovirus to deliver an androgen-responsive reporter gene (McPhaul et al., 1997). The AR function was less than that in normal fibroblasts, mostly as in PAIS, in all the cases examined.

The Androgen Receptor in Cancer

Sex Steroids and Apoptosis

Sex steroids control sex determination during fetal age and ensure further development and differentiation of their responsive tissues at puberty. Along with other hormones and transcription factors, sex steroids regulate and maintain an equilibrium between cellular proliferation and apoptosis (programmed cell death). Sex-steroids' role in apoptosis is evident by the effect of their addition or withdrawal from their responsive

tissues. Androgen ablation by castration causes apoptotic regression of the prostate (Briehl et al., 1991; Colombel et al., 1992). Estrogens inhibit apoptosis in the mammary gland, and ovarian granulosa cells (Cho-Chung, 1978; Billig et al., 1993). Oppositely, androgens enhance ovarian granulosa cell apoptosis (Billig et al., 1993). Thus, unbalanced levels of sex steroids, or abnormalities in their receptors that disturb cellular proliferation-apoptosis equilibrium may lead to the main characteristic of cancer cells: loss of growth control.

Nuclear Receptors and Cancer

The first linkage between cancer and nuclear receptors was for the thyroid receptor. The cellular homologue of v-erbA oncogene (c-erbA) was found to be the α-thyroid receptor (Weinberger et al., 1986; Sap et al., 1986). The α-retinoic acid receptor is implicated in the acquisition of retinoic acid resistance in patients with acute promyelocytic leukemia who have been treated with retinoic acid (Kastner et al., 1992; Roberston et al., 1992). However, the most established link is with receptors for sex steroids. Estrogen receptor's role is well known in breast, endometrial, and vaginal adenocarcinoma (Clarke et al., 1991). Also, several splice variants of the estrogen receptor have been identified in a human meningioma (Blankenstein et al., 1994), and in hepatocellular carcinoma (Villa et al., 1995). In recent years, the role of the AR in cancer has been suggested by the identification of several AR mutations in prostate and male breast cancers.

AR Mutations in Prostate Cancer

Prostate cancer (CaP) is the most common malignancy in aging males. The vast majority of malignant prostatic tumors are androgen-dependent in the early and intermediate stages. Early localized CaP is potentially curable by radical prostatectomy or radiotherapy. However, the only effective treatment for advanced, localized or metastatic CaP is androgen ablation (Santen, 1992). The rate of response to androgen ablation can be as high as 80%, but most tumors progress to androgen-independent

growth after only 12-18 months (Crawford, 1992). One of the mechanisms of progression of CaP growth to androgen-independence is by mutations in the AR that allow the receptor to stimulate the growth of prostate cells despite androgen deficiency. In fact, such mutations have been reported repeatedly in CaP cell lines and tissue from patients (Tilly et al., 1996 and refs. therein). Most of the AR mutations identified in CaP cause a broadening of ligand specificity and/or activation by estrogens, progestins, adrenal androgens, or even antiestrogens in addition to testicular androgens (Veldscholte et al., 1992; Suzuki et al., 1993; Culig et al., 1993; Taplin et al., 1995). For instance, a single amino acid substitution (Thr876Ala) in the ABD of the AR results in receptor that can bind estrogens, progestins, and some antiestrogens in a manner that stimulates tumor cell growth (Veldscholte et al., 1992). More striking, is the frequency of this mutation, identified in the human CaP cell line LNCaP (Veldscholte et al., 1992), in one of 15 CaP cases (Suzuki et al., 1993), and in six of 24 advanced CaP cases (Gaddipati et al., 1994). Another somatic amino acid substitution (Val715Met) in the HBD of the AR allows the mutant AR to transactivate better than the normal AR when it is bound to adrenal androgens and even when it is bound to progesterone (Culig et al., 1993). Mutations of the AR have been found also in advanced prostate cancers prior to hormonal therapy (Tilly et al., 1996). These mutations suggest a selective outgrowth of existing cells with mutant AR rather than the acquisition of new mutations after hormonal therapy, which may explain the rapid progression of prostate cancer to androgen-independent tumors.

However, progression to an androgen-independent growth may not be always the mechanism by which the AR enables prostate tumors to escape androgen-ablation therapy. Amplification and increased expression of a wild-type *AR* has been demonstrated in a subset of hormone-refractory prostate tumors (Koivisto et al., 1997; Visakorpi et al., 1995). The fact that the amplified *ARs* are wild-type and that the levels of their expression is increased, implies that these tumors may still be androgen dependent, and that it is the quantity rather than the quality of the AR that enables tumor cells to grow in the *AR*-amplified tumors.

The role of the ARs with higher transactivity in CaP is also evident by the association of shorter CAG repeats in exon 1 of the *AR* with an increased risk and

younger age-of-onset of prostate cancer (Irvine et al., 1995; Stanford et al., 1997; Giovannucci et al., 1997; Hardy et al., 1996; Ingles et al., 1997). The risk of CaP is higher in African-Americans than Caucasians, which could reflect the shorter CAG repeats in the African-American population (Irvine et al., 1995). In addition, a somatic contraction of this CAG repeat from 24 to 18 has been reported in advanced CaP (Schoenberg et al., 1994). As mentioned previously, shorter CAG repeats of the *AR* increase the transcriptional activation of the receptor (Tut et al., 1997; Kazemi-Esfarjani et al., 1995; Chamberlain et al., 1994).

The human female breasts, unlike related species, develop without the stimulus of copulation or pregnancy. At puberty, ovarian production of estrogen, then progesterone with occurrence of ovulation, induces breast growth and maturation. Thereafter, besides minor repeated stimulation associated with menstrual cycles, breasts are in a 'resting' state until pregnancy. Pregnancy results in a series of changes in the breast that end in the fully differentiated state of lactation. After lactation, breasts return to the 'resting' state. These physiological changes alternate until menopause, where a major regression of breasts occurs. The different stages of breast growth and development require complex control mechanisms that involve a stage-dependent regulation of several genes that are critical for cell proliferation, differentiation, and apoptosis. A defective control system may lead to the transition of the breast normal tissue to breast cancer (BC).

BC is the most common malignancy among women in North America, with a cumulative risk of 12.6%, or one in eight, by age 85. It represents 25-30% of total female cancers, with the number of newly diagnosed cases increasing at a rate of about 1% per year (American Cancer Society, 1994; Marshall, 1993).

Multifactorial Etiology: Stepwise Pathogenesis of Breast Cancer

A gradual transition of the normal breast epithelium to invasive carcinoma is the end result of a stepwise accumulation of multiple genetic changes. Multiple 'hits' are required for such a transition because of the redundancy in the control system that regulate cell proliferation. These genetic aberrations include expression of oncogenes and suppression or loss of anti-oncogenes. Among the constitutional and somatic mutations that lead to or associated with BC are:

- The *p53* locus on 17p in the Li-Fraumeni syndrome (Malkin et al., 1990).

- The HRAS minisatellite locus on 11p (Krontiris et al., 1993).
- Heterozygosity at the locus for ataxia-telangiectasia (Swift et al., 1991).
- Androgen receptor locus on Xq in male BC (Lobaccaro et al., 1993; Wooster et al., 1992).
- The two susceptibility genes: *BRCA1* on 17q in familial breast-ovarian cancer and *BRCA2* on 13q in male and BC (Friend, 1997; Stratton, 1996; Szabo and King, 1995).

The number of these mutations and the sequence in which they occur may vary from one BC to another. However, sex steroid-imbalance is likely to play an important role in nearly all of BCs. Of all BCs, two thirds appear during the postmenopausal period, which is associated with hormonal imbalance, and about one third are hormone-dependent (Henderson and Cannellos, 1980).

BC Susceptibility Genes: BRCA1 and BRCA2

Other than age, family history is the most important risk factor for BC. Of all BC cases, an estimated 5-10% may be due to inherited mutations in one of the two BC susceptibility genes, *BRCA1* and *BRCA2*. In addition to constitutional mutations, several somatic mutations have been found in both genes. However, at least two other genes, p53 and the androgen receptor, are also responsible for inherited predisposition to familial BC. Also, familial cancers other than BC increase its risk by an average of 2.7-fold (Anderson and Badzioch, 1993).

BRCA1, located on 17q, confers susceptibility to early-onset familial breast and ovarian cancers (Miki et al., 1994). The coding region of *BRCA1*, composed of 22 exons, encodes a large polypeptide, 1,863 amino acids, that contain a RING finger motif that is found in a variety of regulatory proteins. *BRCA1* mutations increases the lifetime risk of BC to >80%, and are responsible for ~50% of familial breast cancers and for 75-80% of families with both ovarian and breast cancer. Several somatic and germline mutations have been identified, the majority of which lead to protein truncations. The

position of these mutations correlates with the risk of cancer. While truncating mutations in the 5′ end of the gene are found in families with high fraction of ovarian cancers, families with predominantly or exclusively BCs tend to have mutations in the 3′ end of the gene. This suggested the hypothesis of the existence of a region in the N-terminus of the protein that confers a protection against ovarian cancer (reviewed in Stratton et al., 1996; Szabo et al., 1995).

BRCA2, located on 13q and composed of 27 exons, encodes a large protein of 3418 amino acids, which also contains a RING finger motif. Germline mutations of *BRCA2* are thought to account for ~70% of inherited BCs not linked to *BRCA1* (Tavtigian et al., 1996; Wooster et al., 1994). It confers a lower risk of ovarian cancer and much higher risk of male BC than *BRCA1*. In a study of male BC ascertained in North America, 14% carried *BRCA2* (Couch et al., 1996). However, unlike *BRCA1*, there is no evidence of correlation between the position of *BRCA2* mutations and the risk of male BC. Other cancers such as prostate, pancreas, larynx and ocular melanoma are also at increased frequency in *BRCA2* families (reviewed in Stratton et al., 1996; Szabo et al., 1995).

The biological and biochemical functions of wild-type products of *BRCA1* and *BRCA2* are not yet known. However, the existence of a naturally occurring human 'knockout' of *BRCA1*, a homozygote for the *BRCA1* mutation 2800delAA, with a normal phenotype, apart from early-onset BC, shows that the wild-type product of *BRCA1* is not essential to normal life (Boyd et al., 1995). The majority of mutations, somatic and germline, identified in *BRCA1* and *BRCA2* are protein truncations, likely representing loss-of-function. A wild-type allele of each gene is typically lost or inactivated in the tumors, and overexpression of normal *BRCA1* product into breast and ovarian cancer cell lines inhibits their proliferation (Holt et al., 1996). These data imply that *BRCA1* and *BRCA2* are tumor suppresser genes (reviewed in Stratton et al., 1996; Szabo et al., 1995). Also, besides the RING finger motifs, another similarity between *BRCA1* and *BRCA2* is their binding to Rad51, a DNA-repair protein (Sharan et al., 1997; Scully et al., 1997).

Androgen:Estrogen (A:E) Balance in BC

Ovarian factors were considered important in BC pathogenesis and treatment since 1896, when oophorectomy was shown to cause remission in some BC patients (Beatson, 1896). Over the years, strong epidemiological, clinical, and biochemical evidence suggested the role of endogenous sex hormones, mainly estrogen, in female BC etiology and/or progression (reviewed in Nisker and Siiteri, 1981). The role of estrogen in BC pathogenesis is also evident by the success of anti-estrogen treatment in the management of certain BCs (Pollak, 1996). Anti-estrogens interfere with the binding of estrogens to the estrogen receptor and may actively regulate gene expression in a direction opposite to that of estrogens (Pollak, 1996). Administration of estrogen induces mammary tumors in different animal species (Nisker et al., 1981). High doses of estrogens administered as a therapy for ulcer, recurrent myocardial infarction, and metastatic prostate carcinoma, or in order to attain female characteristics in transsexuals increase the risk of BC in males (Nisker, 1981). Among women, BC is observed only after menarche, and women with higher lifetime exposures to estrogen are at high risk of BC (Akhtar et al., 1983; Pike et al., 1981; Feinleib, 1968). Lifetime exposures to estrogen depend on several reproductive factors such as the age at menarche, first pregnancy, menopause, as well as the number of children.

Male BC accounts for only 1% of all BCs, which could reflect the higher levels of androgens and lower levels of estrogens in men compared to women. In fact, hyperandrogenemic women have breast atrophy, and hypoandrogenic conditions, such as testicular atrophy, infertility, orchitis, and testicular trauma, increase the risk of BC in males (Thomas et al., 1992). The risk of BC in XXY males (Klinefelter syndrome) is 20-fold the risk in normal XY males (Scheike et al., 1973). A fraction of XXY males not only has low circulating androgen levels, but high circulating estrogen levels. This androgen:estrogen (A:E) imbalance could result from increased peripheral aromatization of androgen to estrogen by the female pattern of adiposity seen in some XXY males. Gynecomastia, the unilateral or bilateral enlargement of the male breast, may predispose to male BC (Cutuli et al., 1997), which is not surprising, given the fact that

gynecomastia is also associated with hypoandrogenic conditions and occurs mostly during times of physiological hormonal imbalance (puberty and old age).

Estrogen promotes and maintains normal and tumor breast growth and inhibits cellular apoptosis. Androgen is antiestrogenic. In the ovary, estrogen inhibits ovarian granulosa cell apoptosis, and androgens counteract this effect (Billig et al., 1993). The direct antiestrogenicity of androgen has been also documented repeatedly in certain BC cell lines (Szelei et al., 1997; Couture et al., 1993; Boccuzzi et al., 1993; Hackenberg et al., 1991), and is supported by the advantage of adding androgen to antiestrogen therapy in advanced BC (Ingle et al., 1991).

The mechanism of the inhibitory effect of androgens on BC cell proliferation is poorly understood. A possible mechanism is the induction of cellular apoptosis. Levels of the Bcl-2 proto-oncogene product, which promotes cell survival and inhibits apoptosis, are down regulated by androgens in BC cells (Lapointe et al., 1997). The proto-oncogne Bcl-2 is overexpressed in up to 70% of BCs (Silvestrini et al., 1994), and may be an important negative regulator of apoptosis in these cancers.

If hypoandrogenic conditions are a risk factor for male BC, one might expect that androgen insensitivity (AI) would also be a risk factor (as is the case for gynecomastia). Also, if gynecomastia predisposes to male BC, individuals with gynecomastia due to AIS would be at higher risk of BC. AI in a breast cell disturbs the A:E balance by generating functional hyperestrogenicity, and may increase the risk of cancer in that breast (Fig. 5). AI may result from increased androgen degradation or aromatization to estrogens or from mutations in the AR. Mutant ARs could play a role in the initiation and/or progression of BC either by a loss or a gain-of-function. The loss-of-function could be simply by a loss of the protective effects of androgen, providing a selective advantage for cancer cells. Also, defective ARs that result in AIS may result in increased levels not only of circulating androgen, but also estrogen (Quigley et al., 1995). This may result from reduced feedback inhibition of gonadotropin secretion leading to increased luteinizing hormone (LH) levels, thereby stimulating Leydig cell production of T and estradiol. In fact, it has been shown that serum (Calabresi et al.,

1976) and urinary (Dao et al., 1973; De Giuli et al., 1974) concentrations of estrogens were increased in men with BC, although serum levels of androgens were normal. Another possibility would be the gain of a harmful function that acts to promote BC. For instance, mutant ARs may acquire an ability to bind to estrogen response elements, and thereby transactivate estrogen responsive genes. ARs with DBD-mutations may also be able to superactivate other steroid receptors, possibly by a process of illegitimate heterodimerization (Kazemi-Esfarjani et al., 1993).

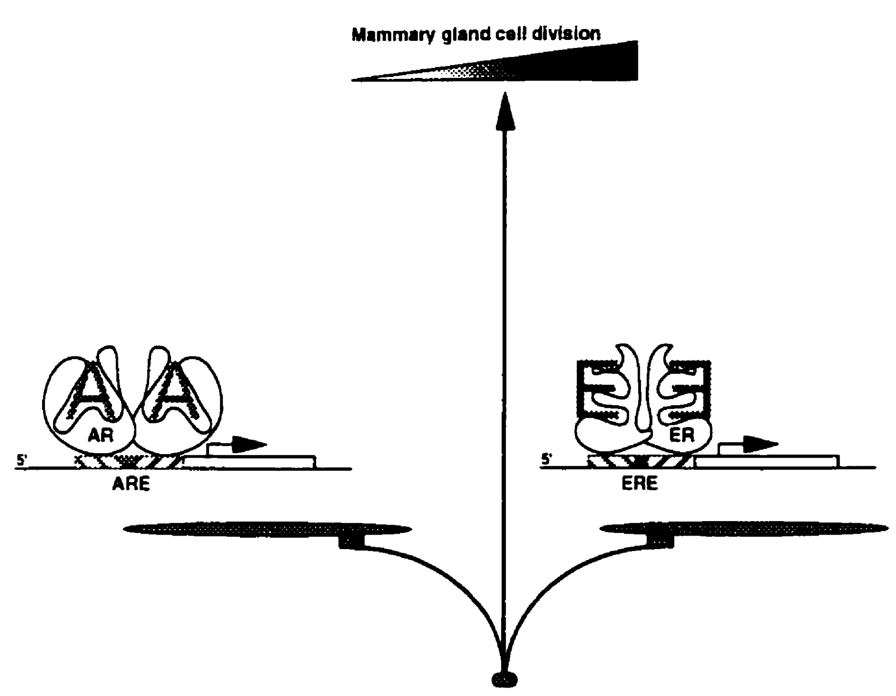

Mammary gland cell division
AR
5'
ARE
ER
5'
ERE

Fig. 5. Androgen:estrogen balance. Functional androgen:estrogen balance depends on the ability to synthesize, respond to, and metabolize androgen and estrogen.

AR Mutations in Male BC

Two reports have described BC in XY males with ambiguous external genitalia due to AR mutations causing PAIS in two unrelated families. In one family, two brothers who had Arg607Gln substitution in the C-terminal Zn finger, developed BC at 55 and 75 years of age (Wooster et al., 1992). This arginine is conserved in AR, ER, GR, and MR. The other report, described a similar substitution Arg608Lys in a 38 year-old man with infiltrative BC (Lobaccaro et al., 1993). This arginine is conserved in all nuclear receptors. If not by chance, the occurrence of the two mutations in conserved adjacent residues in unrelated BC patients implies the role of the respective mutant ARs in BC pathogenesis. However, the mechanism(s) by which such AR mutants may be involved remains speculative. A functional analysis of the two described mutations revealed reduced DNA-binding to an isolated *AREs* and reduced transactivation efficiency for the ARs, but neither binding to *EREs* nor transactivation of ERE-reporter gene was observed (Poujol et al, 1997).

However, besides these two mutations, no association between various degrees of AIS, and male or female BC has been reported! There are several reasons that may explain this fact. First, XY women with CAIS are orchidectomized in early adulthood, in order to avoid the risk of testicular malignancy. The estrogen replacement to promote and maintain secondary female sexual characteristics may carry a lower risk than the normal female estrogenic environment. Second, XY males with PAIS frequently have bilateral mastectomy in early adulthood for the management of gynecomastia. Third, an increased risk of female BC among heterozygotes for *AR* mutations might well go unnoticed in view of the high baseline risk of female BC in the population.

Introduction

A female breast epithelial cell will have a greater chance of becoming neoplastic under the influence of estrogen, if it is also resistant to the antiestrogenic effects of androgen. The fact of random X-chromosome inactivation raises two possibilities: somatic mutations at the *AR* locus on the single active X-chromosome in normal female cancer cells may contribute to an unknown fraction of primary female BC; and females constitutionally heterozygous for any *AR* mutation that causes AI may be hypersusceptible to BC. Androgen receptors are expressed in up to 84% of female BC tumors (Lea et al., 1989); however, the possible association between *AR* mutations and female BC has never been tested.

The length of the polymorphic CAG repeat in exon 1 of the *AR* correlates inversely with the transactivational activity of the AR. To address the question of a possible correlation between CAG-repeat length and BC incidence, I have assessed the length distribution of the CAG repeats of the tumor samples (manuscript 1).

Also, I addressed the question of how often mutations in exons 2-8 of the *AR* in human female cancer cells may contribute to an unknown fraction of primary female BC. A highly sensitive single-strand conformation polymorphism (SSCP) method was adopted to analyze exons 2-8 of the *AR*, which encode the DNA- and androgen- binding domains, in 81 primary postmenopausal breast tumor samples (manuscript 2). Because of the rarity of *AR* point mutations in exon 1 causing AIS, this exon was excluded from the SSCP analysis.

Contributions to the manuscripts

Manuscript # 1. The Polymorphic CAG Repeat of The Androgen Receptor: A Role in Postmenopausal Breast Cancer? Youssef A. Elhaji, Mark Trifiro, and Leonard Pinsky.

Manuscript # 2. The Androgen Receptor gene and Female Breast Cancer. Youssef A. Elhaji, Lenore K. Beitel, Mark Trifiro, and Leonard Pinsky.

My co-authers contributed to this work in the following ways:

Lenore K. Beitel:
Participation in optimizing the conditions for the SSCP analysis and revising manuscript #2.

Mark Trifiro:
My co-supervisor

Leonard Pinsky:
My supervisor
Directing and revising the manuscripts.

The Polymorphic CAG Repeat of The Androgen Receptor: A Role in Postmenopausal Breast Cancer?

Youssef A. Elhaji[1], Mark A. Trifiro[2,5] & Leonard Pinsky[1-5]

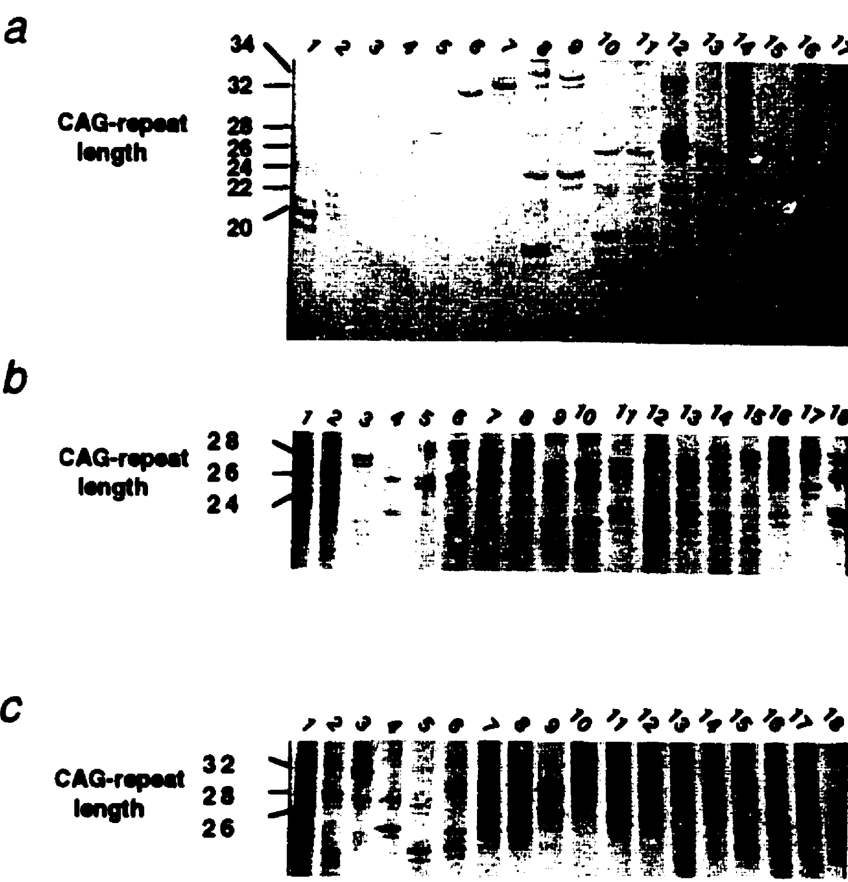
Department of [1]Biology, [2]Medicine, [3]Human Genetics and [4]Pediatrics, McGill University, 1205 Doctor Penfield Avenue, Montreal, Quebec H3A 1B1, [5]Lady Davis Institute of Medical Research, 3755 Cote Ste. Catherine Road, Montreal, Quebec H3T 1E2.

The length of the polymorphic CAG repeat of the human androgen receptor gene (*hAR*) correlates inversely with the transactivational activity of the AR in various types of transfected cells. Decreased AR transactivational activity lowers androgen:estrogen balance, and may thereby effect functional hyperestrogenicity. This may promote the pathogenesis of breast cancer (BC). To investigate whether the occurrence of postmenopausal female BC is associated with longer CAG repeats, we assessed the distribution of CAG-repeat lengths of the *AR* in fresh frozen postmenopausal BC tissues. By the unpaired t-test and the Wilcoxon test, our results show a highly significant shift ($p < 0.0001$) to greater CAG-repeat lengths in BC samples compared to constitutional samples of a composite normal population. Alleles with ≥26 repeats were nearly 3-fold more frequent in the BC samples (32.5% versus 11.7%). The positive association between *ARs* with long CAG repeats and postmenopausal BC suggests that hypotransactive ARs with long polyglutamine tracts have a role in the initiation and/or progression of postmenopausal BC.

In certain diseases, departure of the sex ratio from unity may reflect disparity of sex hormone action. The female:male ratio of breast cancer (BC) is 100:1. BC is observed only after menarche, and women with higher lifetime exposures to estrogen are at higher risk of BC (43, 95). Estrogen promotes and maintains breast development, and inhibits breast cell death (24). Estrogen inhibits ovarian granulosa cell apoptosis, and androgens counteract this effect (11). The direct antiestrogenicity of androgen has also been documented repeatedly in BC cell lines (13, 28, 122); it is supported by the advantage of adding androgen to antiestrogen therapy in advanced BC (62). Hypoandrogenic conditions, such as Klinefelter syndrome and testicular atrophy, increase the risk of BC in males (125). Gynecomastia, which implies hypoandrogenicity, may predispose to male BC (32). The mechanism of the inhibitory effect of androgens on BC cell proliferation is poorly understood. Recently, however, it has been reported that levels of Bcl-2 proto-oncogene product are downregulated by androgen in BC cells (76). Bcl-2, which promotes cell survival and inhibits programmed cell death, is overexpressed in up to 70% of BCs (116).

A deficient or defective androgen-response system disturbs androgen:estrogen balance, and generates functional hyperestrogenicity. The main component of the androgen-response system, the androgen receptor (AR), is an androgen-modulated, DNA-binding, transcriptional-regulatory protein that is a member of the nuclear receptor superfamily (97). One transcriptional domain of the AR, encoded by exon 1 of the X-linked *AR*, contains a polymorphic glutamine repeat that modulates transactivation (66, 97). The length distribution of this CAG repeat in normal populations ranges from 11-33 (38, 47, 64, 118) and correlates inversely with the transactivational activity of the AR (22, 69, 86, 130). Expansions of the CAG repeat to $n \geq 40$ cause spinobulbar muscular atrophy and mild androgen insensitivity in males (69). Relatively short CAG repeats in the normal range are associated with an increased risk and younger age-of-onset of prostate cancer (47, 56, 63, 64, 118). The risk of prostate cancer is higher in African-Americans than Caucasians (64). Contrarily, the overall incidence rates of BC in African-American females are less than in white females (128). These correlations could reflect the shorter CAG repeats in the *ARs* of the African-American population (38, 64). In addition, waist-hip ratio (upper body adiposity), which correlates positively with BC incidence (6, 44), also correlates positively with the CAG-repeat size among female Hispanics (79).

In aggregate, the foregoing data and observations raised the question of a possible correlation between CAG-repeat length and BC incidence. To address this question, we used nested PCR, 10% polyacrylamide minigels, and silver staining, to assess the distribution of CAG-repeat length in the *AR* of postmenopausal BC tissue samples (Fig. 1*a*). Equal-sized alleles were rerun adjacently for confirmation (Fig. 1*b,c*). Each allele yielded a major doublet, with or without minor bands. Only the reproducible major doublets were used to generate the distribution of the CAG-repeat lengths in BC samples (Fig. 1*d*). However, the reproducibility of minor amounts of extra PCR products is very suggestive of somatic expansions. By the unpaired t-test and the Wilcoxon test, our results show a significant ($p < 0.0001$) shift to greater CAG-repeat lengths in BC samples; their mean and median are 24 repeats, compared to 22 in the normal population (Fig. 2). More strikingly, alleles with ≥ 26 repeats were nearly three times more frequent in BC samples.


a
CAG-repeat
length
34
32
28
26
24
22
20
b
CAG-repeat
length
28
26
24
c
CAG-repeat
length
32
28
26

d

control

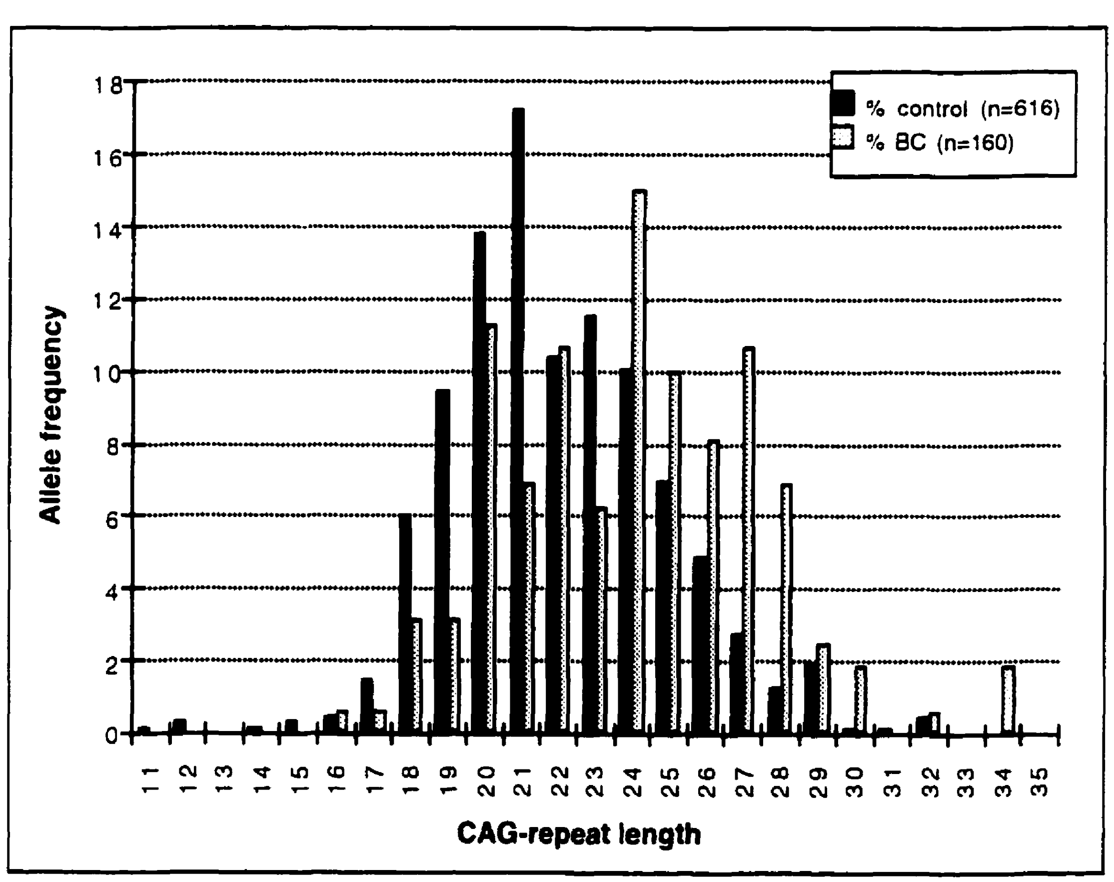
BC samples

28 27 28
24 24 23

CAG-repeat lengths

28
26
24

Fig. 1 Measurement of CAG-repeat lengths. The nested PCR products were separated and sized using 10% polyacrylamide gels and silver staining; ***a***, Known CAG-repeat lengths (20,22,24,26,28,32 and 34 repeats; lanes 1-7 respectively), were used as controls to analyze BC samples (lanes 8-17); ***b***, Equal-sized alleles were rerun adjacently for confirmation [24,26 and 28 repeats as controls (lanes 1-3), BC samples with one of the two alleles sized as: 25 (lanes 4-8), 26 (lanes 9-13) and 27 repeats (lanes 14-18)]; *c*, 26,28 and 32 repeats as controls (lanes 1-3), BC samples with at least one of the alleles with 28 repeats (lanes 4-18); ***d***, As diagrammed here, and as seen in ***a***, each allele yielded a major doublet.



% control (n=616)
% BC (n=160)
Allele frequency
0
2
4
6
8
10
12
14
16
18
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
CAG-repeat length

Fig. 2. The distribution of CAG-repeat lengths in BC samples and in a pooled group of normal white controls [refs. 38 (n=240), 64 (n=39), 118 (n=266), and our laboratory (n=71)]. A significant ($p < 0.0001$) shift to greater lengths was found in BC samples, compared to the pooled group of controls, and to each subgroup, using the unpaired t-test and the Wilcoxon test.

Based on a previous report from our laboratory (69), Giovannucci et. al. (47) estimated that six CAG repeats cause a 12% difference in transcriptional activation. An appreciable decrease in the transcriptional activity of an AR with a long polyglutamine tract could lead to prolonged period of functional hyperestrogenicity in a mammary gland, and this might contribute to initiation and/or progression of BC. Another possible effect of decreased AR-mediated transactivation might be diminished feedback inhibition of testosterone secretion. Appropriately, a recent study showed increased testosterone levels with age in men with longer CAG repeats (67). Two abnormalities in androgen production have been found in women with BC; excessive ovarian production of testosterone in women with either premenopausal or postmenopausal BC, and subnormal production of adrenal androgens in women with premenopausal BC (145). These abnormalities raise the possibility of a difference in the CAG-repeat-length distribution between the two classes of BC, and perhaps, of pathogenetic differences between them. Androgen at high levels (55, 92, 140), or androgen metabolites (54), could have an estrogenic effect in several ways: by promiscuous agonistic binding to the estrogen receptor (ER) as observed in BC tissue and BC cell lines (54, 55, 140); by increased androgen aromatization, an important source of intratumour estrogen production (14, 102); or by virtue of the ability of androgen to competitively displace estrogen from sex hormone-binding globulin (SHBG) (92). Moreover, by negative autoregulation, prolonged androgen stimulation may lead to both decreased AR mRNA levels and functional inactivation of the AR, as described for T47D BC cells (135). Whatever the mechanism(s) of action, it is plausible that decreased AR transcriptional activity could contribute to the hyperestrogenic state of BC tissue. The present data show a positive association between *ARs* with long CAG repeats and BC, and suggest a pathogenic role for ARs with relatively long polyglutamine tracts in the initiation and/or progression of postmenopausal BC.

Further studies are needed to confirm our observation, to assess its quantitative impact on the risk of postmenopausal BC, and to determine its underlying nature. For instance, to address the possibility of somatic mutation as the source of the shift in CAG-repeat-size in BC tissue, we plan to compare CAG-repeat length in matched tumor and normal breast tissues. In addition, our observation predicts that women who are

heterozygous for $(CAG)_n$-expanded *AR* alleles that cause spinobulbar muscular atrophy should be at increased risk. This should also apply to heterozygotes of point-mutated *AR* alleles that cause classical androgen insensitivity (testicular feminization). To our knowledge, no one has data on either group.

Methods

BC samples. 81 fresh frozen breast tumor tissue samples were obtained from the Manitoba Breast Tumour Bank (Dept. of Pathology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada), and stored at -135 °C until processing.

Isolation of Genomic DNA. Genomic DNA was isolated from four 20 μm frozen tissue sections/tumor using TRI-Reagent as recommended by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH, USA).

CAG Repeat Amplification. 3 μl DNA were amplified by two rounds of PCR using nested primers: outer primers (sense: 5'-GTGGAAGATTCAGCCAAGCT-3' starting at nucleotide -25, and antisense: 5'-TTGCTGTTCCTCATCCAGGA-3' starting at nucleotide 339); inner primers, adjacent to the CAG repeat, (sense:5'-CCCGGCGCCAGTTTGCTGCTGCTG-3' and antisense 5'-CTGCTGCTGCCTGGGGCTAGTCTC-3'). The first round consisted of 25 cycles (95 °C/1 min, 58 °C/30 seconds, and 72 °C/1 min) after initial denaturation at 95 °C/2 min. The second round, using 1 μl of the first, consisted of 22 cycles (95 °C/45 seconds, and 72 °C/45 seconds). The PCR reactions were performed in 100-μl reaction volumes. 5% DMSO and a preheated thermal cycler were used to increase the specificity.

Measurement of CAG-repeat length. The PCR products were separated and sized on a 10% non-denaturing polyacrylamide minigel at 37 °C, and then silver stained. Fragments of known CAG repeat sizes (20,22,24,26,28,32, and 34 repeats) were used as a marker ladder. Equal-sized alleles were rerun next to each other for confirmation.

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The Androgen Receptor Gene and Female Breast Cancer

Youssef A. Elhaji[1], Lenore K. Beitel[5], Mark A. Trifiro[2,5] & Leonard Pinsky[1-5]

Department of [1]Biology, [2]Medicine, [3]Human Genetics and [4]Pediatrics, McGill University, 1205 Doctor Penfield Avenue, Montreal, Quebec H3A 1B1, [5]Lady Davis Institute of Medical Research, 3755 Cote Ste. Catherine Road, Montreal, Quebec H3T 1E2.

ABSTRACT

Estrogen promotes and maintains breast development, and higher lifetime 'exposures' to estrogen puts women at higher risk of breast cancer (BC). Androgen is antiestrogenic. The antiestrogenicity of androgen has been repeatedly documented in certain human BC lines. Anything that causes androgen insensitivity (AI) in a cell, affects the functional estrogen:androgen balance by generating a functional hyperestrogenicity in that cell. The main cause of AI is a deficient or defective androgen receptor (AR) activity due to mutations at the X-linked *AR* gene. The AR is expressed in up to 84% of BC tumors, however, *AR* mutations have never been documented in these tumors. To address the question of how often mutations of the *AR* in female cancer cells may contribute to unknown fraction of primary female BC, a highly sensitive single stranded conformation polymorphism (SSCP) method was adopted to detect *AR* gene mutations in 81 BC specimens. Using intronic primers, exons 2-8 of the *AR* of the BC specimens were amplified and analyzed. No mutations were detected in any of the samples.

INTRODUCTION:

Breast cancer (BC) is the most common malignancy among women in North America, with a cumulative risk of 12.6%, or one in eight, by age 85 (2, 85). BC has a multifactorial etiology and stepwise pathogenesis. It results from the accumulation of multiple genetic aberrations including expression of oncogenes and suppression or loss of anti-oncogenes, due to constitutional and somatic mutations. Among these genetic aberrations are mutations at the *BRCA1* locus on 17q in familial breast-ovarian cancer (89), the *BRCA2* locus on 13q in male and female breast cancer (124), the p53 locus on 17p in the Li-Fraumeni syndrome (82), the HRAS minisatellite locus on 11p (73), the androgen receptor locus on Xq in male BC (80, 137) and heterozygosity at the locus for ataxia-telangiectasia (121).

The genetic changes may vary from one BC to another; however, hormonal imbalance may be essential in the pathogenesis of nearly all of them. Of all BCs, two thirds appear during the postmenopausal period, which is associated with hormonal imbalance, and about one third are hormone-dependent (57). Among the pituitary and ovarian hormones, estrogen plays the most important role in normal and tumor breast-tissue proliferation (92). BC is observed only after menarche, and women with higher lifetime 'exposures' to estrogen are at increased risk of BC (43, 95). Estrogen promotes and maintains breast development, and androgens are antiestrogenic. The antiestrogenicity of androgens has been repeatedly documented in certain human BC lines (13, 28, 53, 122), and in advanced BC, the combination of antiestrogen plus androgen therapy is superior to that of antiestrogen alone (62).

Normal breast development requires an optimal estrogenic environment that depends, at least in part, on the estrogen:androgen (E:A) balance within breast cells. A deficient or defective androgen-response system in a cell causes androgen insensitivity (AI) and affects the functional E:A balance by generating a functional hyperestrogenicity in that cell. The main component of the androgen-response system is the androgen receptor (AR). The AR, encoded by the X-linked *AR* gene, is an androgen modulated, DNA-binding, transcriptional-regulatory protein that is a member of the nuclear receptor superfamily (97). The *AR* consists of 8 exons; exon 1 encodes for the transactivation domain, exons 2 and 3 code for the DNA-binding domain and exons 4-8 for the androgen-binding domain.

ARs are expressed in up to 84% of female BC tumors (78), however, their role in maintaining E:A balance in these tumors is not fully understood. We hypothesize that females who are heterozygous for mutations at the *AR* locus are likely to be at higher risk of BC due to the subpopulation of breast cells in which the mutant allele is borne by the single active X chromosome. In our previous study (39) we reported a positive association between longer CAG-repeats in exon 1 of the *AR* , which decrease the transactivational activity of the AR (22, 69, 86, 130), and the occurrence of postmenopausal BC. In the present study, we investigated whether other *AR* mutations are associated with BC.

MATERIALS AND METHODS

BC Samples. 81 fresh frozen breast tumor tissues were obtained from the Manitoba Breast Tumour Bank (Dept. of Pathology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada), and stored at -135°C until processing.

Amplification of Genomic DNA. Genomic DNA was isolated from four 20 μm frozen tissue sections/tumor using Tri-Reagent as recommended by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH, USA). PCR amplification of individual exons 2,3 and 5-8, and two overlapping fragments of exon 4 (4I and 4II) of the *AR* from each specimen along with normal and known AR mutants as control were performed in 100 μl reaction volumes.

Intronic primers used for DNA amplification:

Exon	Primer	Sequence	Expected fragment-length
2	2A	GTCATTTATGCCTGCAGGTT	314 bp
	2B	TCTCTCTCTGGAAGGTAAAG	
3	3A	TCAGGTCTATCAACTCTTGT	192 bp
	3B	GGAGAGAGGAAGGAGGAGGA	
4I	4IA	ATTCAAGTCTCTCTTCCTTC	237 bp
	4IB*	CAAAGGAGTCGGGCTGGTTGTT	
4II	4IIA*	GACAGTGTCACACATTGAAGGCTATG	248 bp
	4IIB	GCGTTCACTAAATATGATCC	
5	5A	GACTCAGACTTAGCTCAACC	277 bp
	5B	ATCACCACCAACCAGGTCTG	
6	6A	CAATCAGAGACATTCCTCTGG	267 bp
	6B	AGTGGTCCTCTCTGAATCTC	
7	7A	TGCTCCTTCGTGGGCATGCT	263 bp
	7B	TGGCTCTATCAGGCTGTTCTC	
8	8A	ACCTCCTTGTCACCCTGTTT	295 bp
	8B	AAGGCACTGCAGAGGAGTAG	

* exonic primer

SSCP Analysis. Based on a combination of two SSCP methods (93, 101), a highly sensitive SSCP method was used in this study. 1-2 μl of the PCR products were denatured in 5 μl DMSO buffer (30% DMSO, 1mM EDTA, 0.05% Xylene Cyanol, and

0.05% Bromophenol Blue) at 95°C for 5 min. then chilled immediately in ice water. The samples were loaded on non-denaturing 10% polyacrylamide minigels (90X80X1mm) with 1.3% cross-linking, and 10% and glycerol. A tris/glycine buffer (0.05M tris, 0.38M glycine, pH 8.3) was used as gel and running buffers. Electrophoresis was performed in SE 260 dual cooled elcctrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) for 3-5 hrs. at 4°C. The gels were silver stained using Silver stain plus kit (Bio-Rad Laboratories, Mississauga, ON)

Preserving and Drying Gels. The gels were soaked in a 10% glycerol, 45% methanol and 10% acetic acid solution for 20 min. Then dried in cellophane sheets in a fume hood for 1-2 days.

RESULTS and DISCUSSION

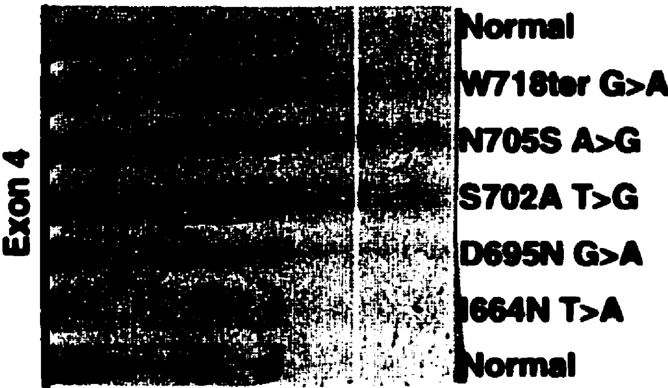
Female BC is 100 times more frequent than male BC, which could reflect the high levels of estrogen and lower levels of androgen in women compared to men. In fact, high doses of estrogens administered as a therapy for ulcer, recurrent myocardial infarction, and metastatic prostate carcinoma; or in order to attain female characteristics in transsexuals, increase the risk of BC in males (92). Likewise, clinical conditions that result in hypoandrogenism, such as Klinefelter syndrome and testicular atrophy, appear to increase the risk of BC in males (125). If hypoandrogenic conditions are risk factors for BC, one would expect the same for AI due to AR mutations. Indeed, there are two reports of BC in adult males with partial AI due to constitutional point mutations affecting adjacent residues, (Arg607Gln and Arg608Lys substitutions) in the DNA-binding domain of the AR (80, 137).

In this study, no mutations were detected in exons 2-8 of any of the samples. Exon 1 was excluded from the analysis because of the vast majority of *AR* mutations that cause AI are in exons 2-8 (48). To increase the sensitivity and reliability of SSCP analysis, a combination of two methods was used for this study (39, 101). However, the efficiency was low for exon 4 due to fragment length (370 bp), and for exon 7, probably

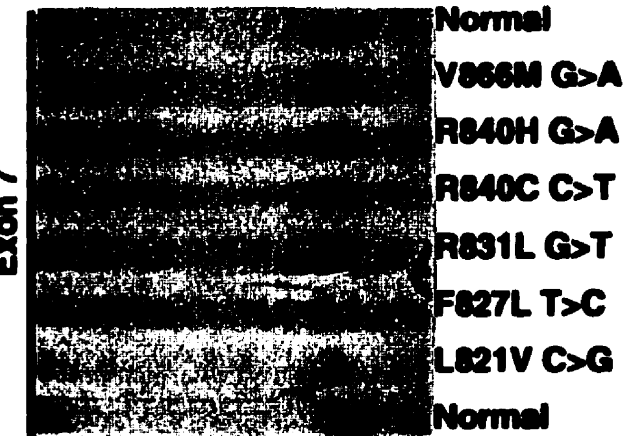
due to the stability of its single-strand configuration. Thus, two overlapping fragments were used to analyze exon 4, and direct sequencing of exon 7 of 20 samples was performed. Using known AR mutations previously described by our laboratory, the sensitivity of the analysis performed in this study was estimated to be >90% (fig. 1). Therefore, the negative results of this study are unlikely to be due to technical problems.

The results of this study show no evidence for the hypothesis that mutations of the DNA- or androgen-binding domains play a role in the possible loss of the protective effect of androgens in female BC tissue.

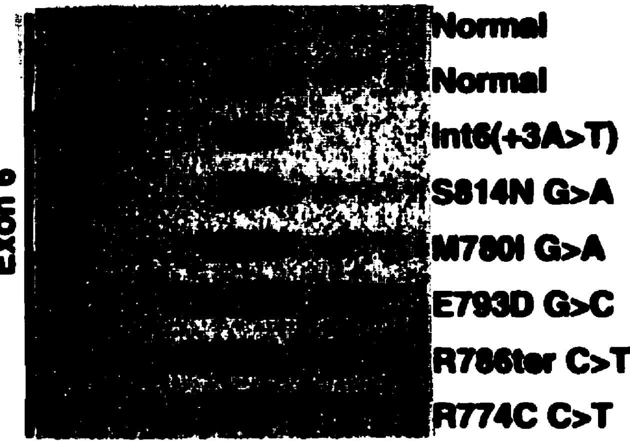
If in fact BC is associated with decreased AR transactivation due to longer polyglutamine-tracts (or other mutations in the transactivational domain), but not with mutations in the androgen- and DNA-domains of the AR, this would raise the possibility that mutant ARs with decreased transactivation may play a role only if they retain normal DNA-binding. It has been shown that N-terminal AR mutants exert a dominant-negative activity on other steroid receptors that bind to the same *HREs* (139). This dominant-negative activity requires DNA binding. In fact, it has been observed that when the wild-type AR is coexpressed with glucocorticoid or progesterone receptor the resultant transcriptional activity approaches the transcriptional activity of the less potent receptor (139). Thus, by retaining the ability to bind DNA, polyglutamine-expanded AR variants may repress the transactivation of other transcription factors. This effect, together with decreased AR transactivation, may combine and increase the risk of postmenopausal BC.



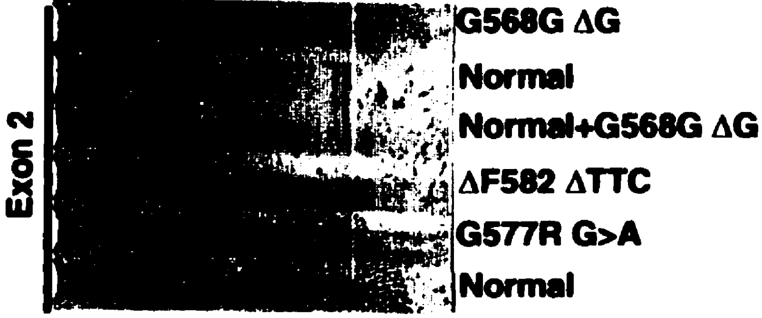
Exon 4
Normal
W718ter G>A
N705S A>G
S702A T>G
D695N G>A
I664N T>A
Normal



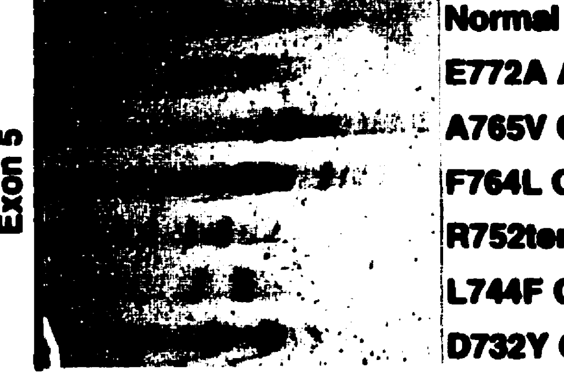
Exon 7
Normal
V866M G>A
R840H G>A
R840C C>T
R831L G>T
F827L T>C
L821V C>G
Normal



Exon 6
Normal
Normal
Int6(+3A>T)
S814N G>A
M780I G>A
E793D G>C
R786ter C>T
R774C C>T



Exon 2
G568G ΔG
Normal
Normal+G568G ΔG
ΔF582 ΔTTC
G577R G>A
Normal



Exon 5
Normal
E772A A>C
A765V C>T
F764L C>G
R752ter C>T
L744F C>T
D732Y G>T

Fig. 1. Detection of AR mutations using PCR-SSCP analysis. Using known AR mutations, the sensitivity of the combined SSCP method was estimated to be >90%. Individual exons were analyzed using intronic primers, 10% polyacrylamide minigels and silver staining.

ACKNOWLEDGMENTS

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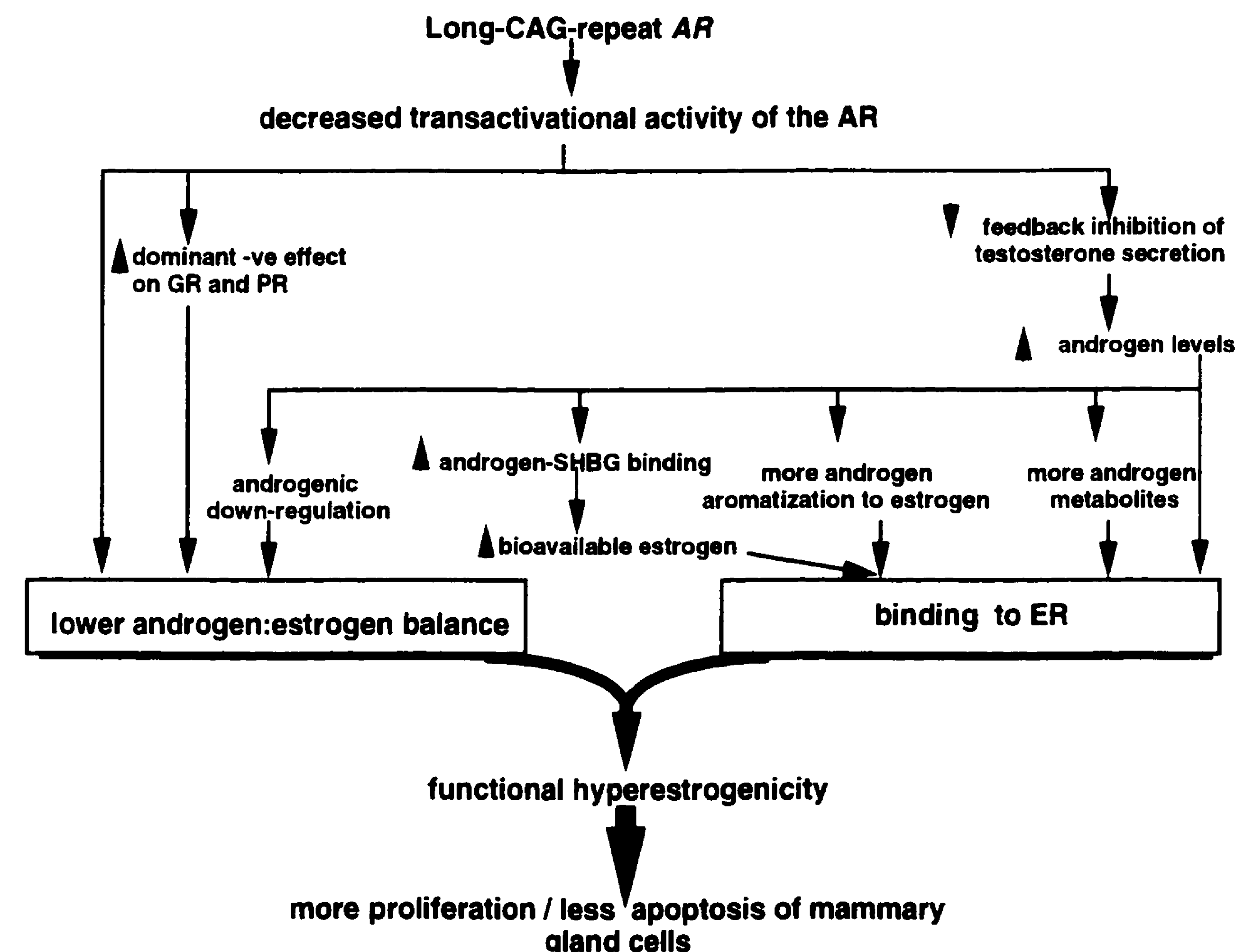
To mediate the diverse effects of androgens in different tissues, the actions of the AR must be cell- . age- and stage-specific. It is, hence, not surprising that different AR mutations cause different disorders. *AR* mutants could lead to: a decreased transactivational activity of the AR resulting in AIS, possibly associated with BC; a gain of function, superimposed on a loss of function, resulting in SBMA; or an increased transactivational activity of the AR associated with CaP.

To investigate the role of the AR in postmenopausal BC, I used the SSCP technique to screen exons 2-8 of the *AR* in 81 BC samples for point mutations to evaluate the DNA- and androgen-binding domains. No mutations were detected. Also, I used 10% polyacrylamide mini-gels to assess the length distribution of the polymorphic CAG-repeat, thereby evaluating the transactivational activity of the AR. The length distribution of the polymorphic CAG repeats showed a significant ($p < 0.0001$) shift to greater lengths in BC samples. My results suggest a pathogenic role for ARs with relatively long polyglutamine tracts in the initiation and/or progression of BC. However, there was no evidence that point mutations in the binding-domains play such a role .

Decreased AR transactivational activity due to long Gln repeats may effect functional hyperestrogenicity in several ways (summarized in fig. 6), and thereby, could promote the initiation and/or progression of BC.



Long-CAG-repeat *AR*
decreased transactivational activity of the AR
dominant -ve effect on GR and PR
feedback inhibition of testosterone secretion
androgen levels
androgenic down-regulation
androgen-SHBG binding
more androgen aromatization to estrogen
more androgen metabolites
bioavailable estrogen
lower androgen:estrogen balance
binding to ER
functional hyperestrogenicity
more proliferation / less apoptosis of mammary gland cells

Fig. 6. **Possible mechanisms by which long CAG repeats of the *AR* may participate in initiation and/or progression of BC.**

The positive association of relatively long CAG repeats and female BC tissue predicts that women who are heterozygous for *AR* alleles with CAG-repeat expansions causing SBMA should be at higher risk of BC. Gynecomastia, which predisposes to BC, is often the earliest manifestation of SBMA and appears in more than half the patients (Quigley et al., 1995). However, SBMA is a rare condition which may account for the lack of evidence for such increased risk.

The results of this study suggest a possibility of a similar association between longer CAG repeats of the *AR* and male BC. In a recent study, screening of exons 2-8 of the AR in male BC tissue samples revealed no mutations (Hiort et al., 1996); however, the CAG-repeat length distribution was not assessed.

If there is a positive association between female BC and a decreased AR-mediated transactivation due to longer CAG-repeats, one would expect an even greater association between BC and AR mutations in the binding domains. Against all expectations, no such mutations have been detected, either in female BC (my study) or in male BC (Hiort et al., 1996). In order to reconcile these apparently discordant data; it is tempting to speculate that the role of the CAG-expanded, hypotransactive AR in BC may require normal androgen- and DNA-binding. By retaining the ability to bind DNA, such AR variants may prevent alternative transcription factors from exerting their protective effect against BC. For example, N-terminal deletion mutants as well as wild-type ARs exert a dominant-negative activity on other steroid receptors that bind to the same *HREs* (Yen et al., 1997), and this dominant-negative activity requires DNA-binding. In fact, it has been shown that the resultant transcriptional activity of coexpressed wild-type steroid receptors that bind to a common *HREs* will approach the transcriptional activity of the less potent receptor (Yen et al., 1997). Interestingly, this dominant-negative effect is augmented by the addition of testosterone. Given all this, and the tendency for women with postmenopausal BC to have high testosterone levels (Dorgan et al., 1996; Zumoff et al., 1994; Secreto et al., 1994), a longer glutamine tract of the AR could be more effective in promoting tumor growth than

an AR with a point mutation in its DBD or LBD. This would also help to explain why BC has never been reported in heterozygotes for AIS, who are likely to have an appreciable subpopulation of cells in which the active X chromosome bears the mutant AR allele, and who are under the influence of high levels of estrogens. On the other hand, XY hemizygotes are likely to be protected due to their low levels of estrogens.

Finally, the improved techniques described in this study have several advantages including the elimination of the hazards involved in using radioactive materials, easy handling of the mini-gels, low cost and increased sensitivity. For the SSCP technique I have combined the advantages of two highly sensitive methods, which can be used effectively in screening for point mutations in large genes and/or large numbers of samples. The technique I used in assessing the CAG-repeat lengths can be preferably used in detecting the different alleles not only of the *AR* gene, but also of other genes that contain polymorphic repeats and also in detecting small insertions and/or deletions.

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