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14. ABSTRACT

In mice in which human androgen receptor (AR) replaces the endogenous murine gene, variation in the length of a polymorphic N-terminal polyglutamine tract affects initiation, progression and therapy response of prostate tumors in the TRAMP mouse model. This provides a genetic paradigm in which to dissect AR functions that determine response to treatment. We developed a mouse model that more accurately reflects human disease, utilizing ETV1 overexpression and heterozygosity for PTEN loss, coupled with variation of the androgen axis via AR alleles differing in Q tract length. In vitro, Q tract length was shown to influence ligand-independent AR activation – shorter Q tract lengths led to greater phosphorylation of a site in the AR hinge region, enhancing both transactivation and nuclear shuttling of the receptor. The new tumor model proved milder than expected, with a lethal phenotype occurring only at late age in a subset of mice, perhaps similar to PCa occurrence in man. Gene expression profiles in this model showed effects of ETV1 prior to any notable pathophysiology, supporting the notion of ETV1 overexpression as an early oncogenic event. The expression patterns revealed a strong antagonism between AR and ETV1 for a subset of AR target genes, and this antagonism was abrogated in a background of PTEN loss. Antagonism at the molecular level may lead to synergy at the disease level and may be subject to variation in AR allelic strength and androgen levels. Further knowledge of these variables may lead to better predictors for response to androgen ablation therapy.

15. SUBJECT TERMS
androgen receptor, polyglutamine tract, mouse models, response to therapy

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Humanized Androgen Receptor Mice: A Genetic Model for Differential Response to Prostate Cancer Therapy

INTRODUCTION

Androgen acting via the androgen receptor (AR) is required for prostate cancer (PCa) initiation and progression. Androgen ablation therefore successfully combats metastatic disease, but in only a few years tumors progress to hormone-resistant lethal growth. Even with new treatments that more completely block androgen synthesis and more effectively antagonize AR action, resistance occurs in a subset of patients (2). Therefore to date, AR remains the primary therapeutic target in PCa. To study aspects of AR in human disease, we previously "humanized" the mouse AR gene by germ-line recombination (3), creating three *h/mAr* alleles varying in length of a polymorphic N-terminal glutamine tract (Q tract) that is associated with male phenotypes. Q tract length correlates inversely with AR transcriptional strength *in vitro* (4). In the aggressive TRAMP mouse PCa model, the short 12Q tract leads to earlier initiation but slower tumor growth that responds well to androgen ablation, whereas the longer 48Q AR has delayed initiation but progresses rapidly to castration resistance (5, 6). The goal of this project was to develop a mouse model that more accurately reflects human disease in order to characterize gene expression changes that correlate with androgen depletion and response to this therapy. Such genes would serve as candidate biomarkers of response to treatment in man. AR allelic variation was used to elicit subtle differences in strength of the androgen axis since such variation showed significant effects in castration response in the TRAMP model, and as we have shown more recently, in the normal prostate response to castration and to restimulation with androgen as well (7). The genetic tumor model utilized *Pten* deficiency, frequent in PCa, and overexpression of *Etv1*, a frequent partner in TMPRSS2-ETS fusions (11). This model proved milder than anticipated, with the lethal phenotype occurring only at late age and in a minority of mice. This perhaps indeed more accurately reflects human disease, in which only a subset of men evidence aggressive disease. Thus while the model diverged from its intended progression, intriguing results were obtained that may be more informative for initiation of prostate cancer and early rather than late disease. This may ultimately help to distinguish indolent from lethal cancers.

BODY

The grant outlined four tasks in the Statement of Work. We have completed the first using a modified genetic strategy. Significant progress on the second was reported previously and so only briefly mentioned here; additional experiments will be performed prior to writing up this study for publication. The last year has focused on Tasks 3 and 4. Histopathological analysis has been performed for all mouse samples to determine grade of disease and expression of key markers (e.g., AR, AKT) by immunohistochemistry, as well as gene expression profiling, by both microarray and RNA-seq methodology. Gene expression validation by qRT-PCR has been accomplished for several indicator genes. In Task 4, RNA-seq data from mice were translated into ONCOMINE to identify relevant human pathways. Novel effects discovered in AR regulation due to interaction with ETV1 are currently being pursued at a mechanistic level in human prostate cancer cell lines.

Task 1. Establish mouse lines with both conditional PTEN and Q-tract variant humanized AR (h/mAr) alleles.

All animals were generated, aged and samples obtained. We initially proposed conditional PTEN deletion (8) to initiate prostate tumorigenesis in C57BL/6 mice carrying each

humanized AR allele (*hAR^{12Q}, hAR^{21Q}, hAR^{48Q}*) (3). Early in the project this strategy was modified to initiate cancer by global inactivation of one PTEN allele and cooperation with a prostate-targeted ETV1 transgene (9-11), on a FVB genetic background. This approach was chosen to provide more uniform oncogenesis than cre-mediated prostate-specific deletion of PTEN and for reduced resistance to oncogenesis on the FVB background. Approval for the modified procedure and animal use was obtained from U-M's UCUCA and from the DOD MRMC Animal Use Committee.

For each Q-tract allele, 3 groups (N = 5 - 13) were compared - **pretreatment** (12 weeks of age), **untreated** (intact at ~ 24 weeks), and **treated** (castrated at 12 weeks, aged to ~ 24 weeks). An equivalent number of littermate controls lacking the ETV1 transgene were compared. This model proved to be less aggressive than anticipated at the originally planned 6 months (24 wk) time points, so mice were aged until moribund or until lymphomas (due to Pten deficiency (12)) necessitated sacrifice, with an average age at sacrifice of 43 weeks (intact range 22 - 83 weeks; treated range 20 - 56 weeks), except for the pretreatment group that was sacrificed at 12 weeks. At sacrifice, prostates were excised, dissected into individual lobes (ventral, dorsal-lateral and anterior) and one of each pair of lobes processed for histology and the other for molecular analysis.

Task 2. Determine the role of the Q-tract in ligand-independent AR activation in vitro

ARs with shorter Q tracts are hypersensitive to hormone levels, as we demonstrated in mice at a systemic level (7), and in the TRAMP tumor model (5). In particular, ARs with short Q tracts are more active at low ligand levels than are ARs with long Q tracts. To get at the underlying molecular mechanism, AR expression vectors were transfected into prostate cell lines. Ligand-independent activation of AR by growth factor, modeled by co-expression of constitutively active Raf-1 kinase, was sensitive to Q tract length and appeared to correlate with the phosphorylation status of a serine residue, S650, in the AR hinge region. Phosphomimetic mutants (AR-S650E) demonstrated a pronounced influence on AR activity at low as well as high hormone levels and this activity inversely correlated with Q tract length. Further, this activity influenced efficiency of nuclear localization. Together with data from other labs (13-15), this suggests that Q tract length influences AR transcriptional activity at multiple levels, including nuclear shuttling, coactivator recruitment, and efficacy of ligand-independent activation. While these genetic effects do not significantly affect risk of disease, they may nevertheless influence course of disease and response to androgen deprivation therapy (6, 16).

Task 3. Determine the molecular correlates of androgen ablation response as affected by AR strength in PTEN-deficient mice with prostate-specific transgenic ETV1 expression

After some delays in breeding and a need for longer time points due to the slow progression of the model, all prostate samples were obtained. Tumorigenesis in this model relies on heterozygous inactivation of the tumor suppressor PTEN (*Pten$^{+/-}$*), coupled with a "second hit" in half of the mice provided by a prostate-targeted human ETV1 transgene (*ETV1-Tg^{+}*) (11, 17). All mice also carried alleles of humanized AR with short, median or long Q tracts (*hAR^{12Q}*, *hAR^{21Q}* or *hAR^{48Q}*, respectively) (3) and were either left intact for the duration of the experiment or castrated at 12 weeks to test response to androgen ablation. Mice were aged until moribund or until lymphomas (due to *Pten* deficiency (12)) necessitated sacrifice, with an average age at sacrifice of 43 weeks (range: 20 - 83 weeks). Hematoxylin and eosin (H&E) stained slides were prepared from prostate lobes from all mice and were examined by Dr. Michael Ittmann (Baylor University), our collaborating pathologist, who scored samples from each mouse for cellular abnormalities including grade of PIN and evidence of adenocarcinoma.

RNA was extracted from the second lobe from each pair for molecular analysis. Due to modest extent of disease in the first samples examined, we decided that the originally planned laser capture microdissection (LCM) would not be sufficiently informative to justify the much greater effort and expense. Instead, RNA was extracted from individually frozen whole prostate lobes for analysis. As described below, molecular effects from ETV1 and PTEN were still observed.

Cooperation between overexpression of the ETS transcription factor ERG with PTEN deficiency in promoting prostate tumor progression in mice has been previously reported (9, 10). It is currently unknown whether ETV1, another ETS factor family member, synergizes with PTEN deficiency in a similar manner. Here, the majority of intact *Pten*$^{+/-}$ mice developed prostatic intraepithelial neoplasia (PIN), a precursor lesion, and among intact mice progression to adenocarcinoma (ADC) was only seen in mice also carrying the *ETV1* transgene, *although* overall PIN rates were similar to non-transgenics (Fig. 1). This observation suggests that PTEN

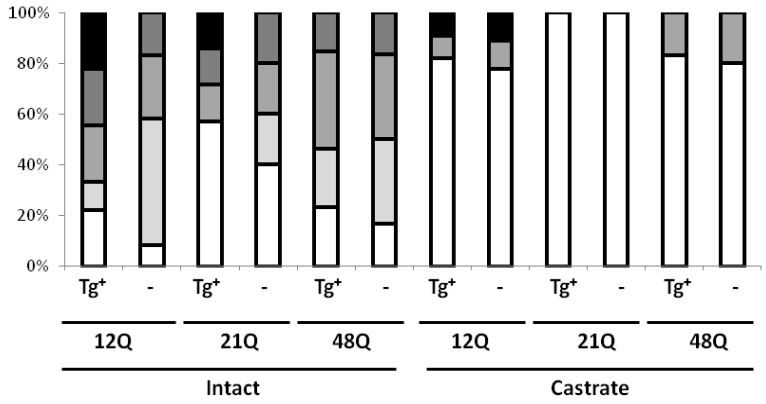

Fig. 1 - Prostate pathology of *Pten*$^{+/-}$ mice. Dorsolateral (DLP) and ventral (VP) prostate lobes were formalin-fixed and paraffin-embedded (FFPE), hematoxylin and eosin (H&E) stained, and examined by our collaborating pathologist, Dr. Michael Ittmann. Scoring was normal (N), PIN stage 2-4 (PIN2-4) or adenocarcinoma (ADC). The proportion of mice per group at each stage is shown in the bar graph. The graph summarizes the most advanced disease observed in either prostate lobe within each mouse. N = 111 total mice.

deficiency and ETV1 expression may cooperate to enhance PCa progression, but not PIN development. Most castrated mice showed prostate atrophy due to androgen ablation, and there was a low rate of PIN and ADC in castrated mice, revealing hormone-refractory disease onset and progression in this model. Rates of PIN and ADC were similar in castrated mice regardless of the ETV1 transgene, but ADC was seen exclusively in *hAR*^{12Q} mice. Interestingly, 4/5 cases of ADC were observed in *hAR*^{12Q} mice and 1 case in *hAR*^{21Q}, indicating that AR strength corresponding to length of the Q-tract may play a role in disease progression, as predicted based on the TRAMP model (5). However, in this model *hAR*^{12Q} mice appear to progress more quickly, while in TRAMP the *hAR*^{12Q} tumors occurred earlier but remained more well-differentiated (5). Also, *hAR*^{21Q} mice showed less overall disease than *hAR*^{12Q} or *hAR*^{48Q} in intact and castrate groups, suggesting that the median AR Q-tract length may be optimal for balancing AR-driven proliferation and differentiation signals in hormone-dependent as well as hormone-refractory disease. Representative histopathology is shown in Fig. 2.

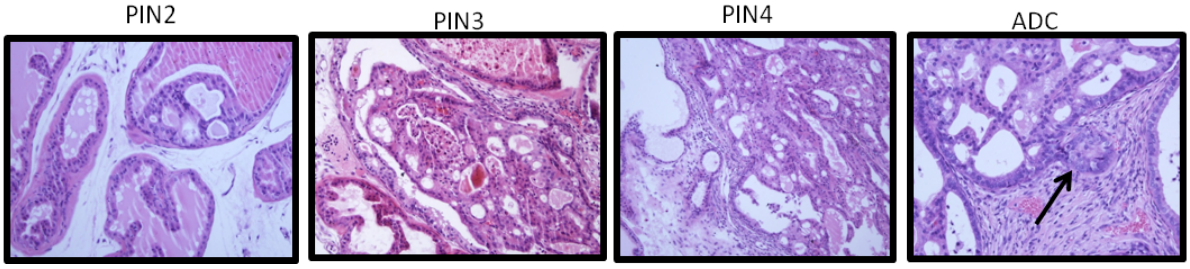

Fig. 2 - Histopathology in *Pten*$^{+/-}$ mice. Representative H&E-stained prostate sections from each disease stage are shown. In PIN2-4 uncontrolled epithelial cell growth is apparent, but the basement membrane remains intact. In ADC the epithelial cells can be seen invading the surrounding stroma (black arrow).

Immunohistochemical (IHC) staining revealed AR protein expression in prostate epithelial cells in all stages of disease (Fig. 3). This observation mirrors human PCa, where AR is generally expressed throughout disease progression, including hormone-refractory disease (18). Phospho-Akt (pAkt) staining was present exclusively in PIN and ADC foci, including PIN2 (Fig. 3), implicating inactivation of the remaining *Pten* allele as a relatively early event in disease progression in this model. The phosphatase PTEN exerts its tumor suppressive function by dephosphorylating PI3-Kinase (PI3K), thus inhibiting PI3K signaling, which in turn prevents phosphorylation and activation of AKT, which can promote cell growth and an androgen-independent phenotype (19, 20). Here, positive pAKT staining suggests that the remaining *Pten* allele has been inactivated allowing activation of the PI3K/AKT pathway and promoting PIN (Fig. 3). This observation parallels the frequent loss of *Pten* in human PCa (10, 21).

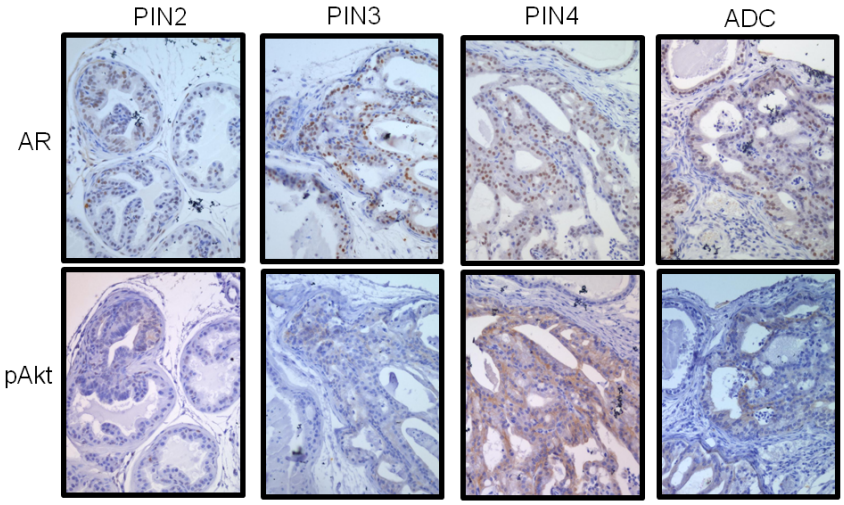

Fig. 3 - IHC staining in *Pten*$^{+/-}$ mice. FFPE prostate sections from *Pten*$^{+/-}$ mice were immunostained for either AR or pAkt protein. Representative slides are shown. Epithelial AR remains expressed in all stages. pAkt is only observed in PIN/ADC foci. Adjacent normal glands lacking pAkt are visible, even in samples with PIN4 and ADC.

We previously measured gene expression in 24 week old *ETV1-Tg*$^{+}$ (*Pten*^{wt}) mice by microarray to determine the molecular pathways altered by the transgene, and reported some of these findings last year. Despite a mild phenotype (PIN evident in 20% of *hAR*^{12Q} and *hAR*^{48Q} *ETV1-Tg*$^{+}$ mice), the transgene induced global changes in gene expression (Fig. 4).

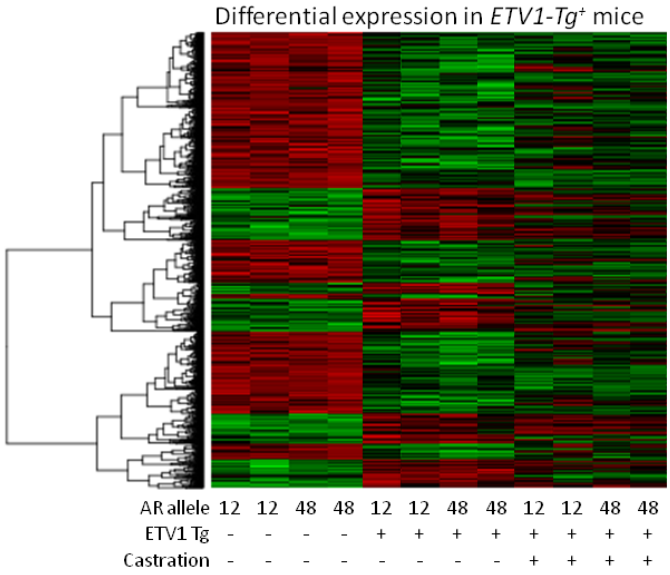

Fig. 4 - Androgen-regulated genes are dysregulated in *ETV1-Tg*$^{+}$ mice. Gene expression microarray analysis reveals global changes in gene expression in prostates of *ETV1-Tg*$^{+}$ mice. 2 pools of RNA per each of the 6 genotype/treatment groups were analyzed on Affymetrix Mouse 430 2.0 microarrays at the University of Michigan Microarray Core. Shown on the heatmap are the top differentially expressed genes ($p < 0.001$) between intact *ETV1-Tg*$^{+}$ and *wt* mice. Rows represent genes (clustered by expression pattern), and each column represents a pool of RNA. Red and green indicate high and low expression, respectively. Dr. Jun Li (University of Michigan) assisted with bioinformatics data analysis.

In order to compare results across high-throughput platforms, and to establish novel deep seqeuncing methodology for future studies in our lab, these samples were also analyzed by RNA-seq. Briefly, total RNA was pooled from samples from each genotype/treatment group, mRNA was purified from total RNA, converted to cDNA, and barcoded sequencing adapters were ligated. The sequence barcodes allowed all libraries to be sequenced together to minimize technical variability. Sequencing was carried out on the Illumina HiSeq at the University of Michigan sequencing core. Sequences were aligned to the transcriptome, and counts per gene were used to calculate differential expression. Dr. Jun Li (University of Michigan) and his lab members generously provided server access for data storage and analysis, and assistance with programming and bioinformatics. Data sets from microarray and RNA-seq showed significant overlap in genes identified as differentially expressed, but as expected the RNA-seq data was deeper and had greater statistical significance.

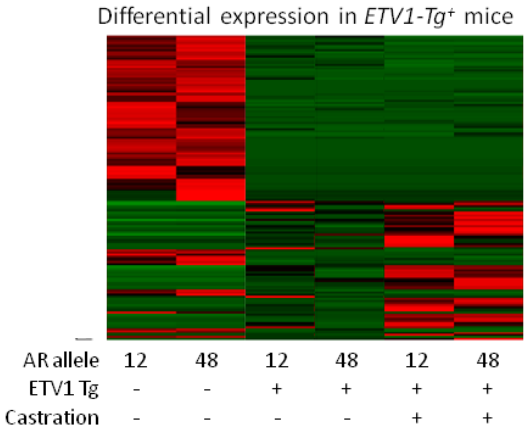

Fig. 5 - RNA-seq analysis confirms differential expression in *ETV1-Tg*$^+$ mice. RNA samples from each genotype/treatment group were pooled into a single barcoded sequencing library per group, and all pools were sequenced simultaneously. The heatmap shows differential expression in *ETV1-Tg*$^+$ mice. Although the genes cluster slightly differently than in the microarray heatmap, there was significant overlap between the genes identified by both platforms.

Many canonical androgen-regulated genes showed differential expression by microarray and RNA-seq. Gene expression patterns in *ETV-Tg*$^+$ mice were validated by qRT-PCR (Fig. 6). Expression of representative androgen-dependent genes *Nkx3-1*, *Pbsn* and *Tmprss2* was abolished by castration, as expected, but also was severely reduced by the ETV1-Tg. The androgen-repressed gene *Clu* shows the complementary pattern; a sharp increase in expression following castration but also an increase from the ETV1-Tg (Fig. 6).

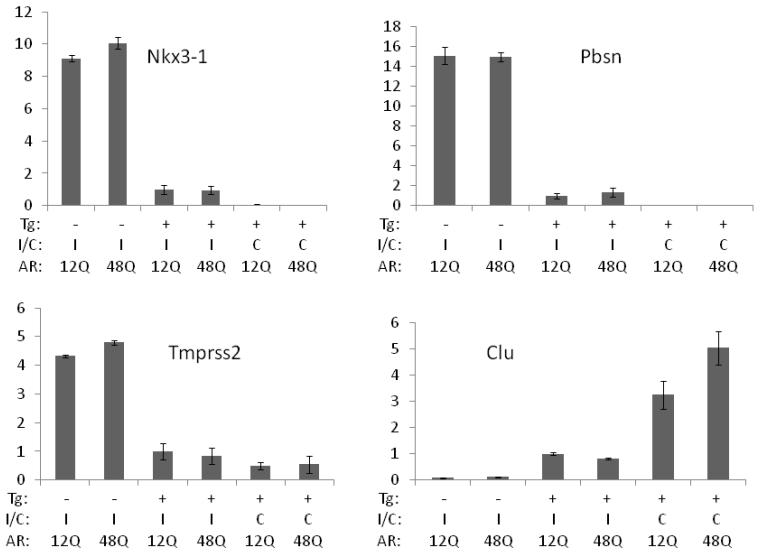

Fig. 6 - qRT-PCR validates repression of androgen-regulated genes. The expression patterns of representative androgen-regulated genes in *wt*, *ETV1-Tg*$^+$ and castrated mice were validated by qRT-PCR. Androgen-induced genes *Nkx3-1*, *Pbsn* and *Tmprss2* are repressed by the ETV1-Tg as well as by castration. *Clu*, which is normally upregulated following castration, is also upregulated in *ETV1-Tg*$^+$ mice. Bars represent mean relative expression +/- SEM.

Overall this pattern suggests that ETV1 overexpression, which occurs in 10-15% of human PCa (22), may be antagonizing AR and suppressing the normal androgen-regulated gene expression program. In particular the *Nkx3-1* gene, a tumor suppressor frequently lost in human PCa (23, 24), shows a sharp reduction in expression with the ETV1-Tg. Direct antagonism of AR transactivation by the ETS family member ERG has been reported in genome-wide studies (25, 26), but has not yet been reported for ETV1. Reports examining the interaction of ETV1 and AR at limited genomic targets suggest that they may cooperate to induce AR target gene expression (17, 27), but broader interaction has not yet been reported. AR and ETV1 binding sites overlap frequently in the genome (28), and the proteins are known to physically interact (17), so it is plausible that they interact directly at many targets. Future work, initiated below in the context of Task 4, will address the mechanism of interaction of AR and ETV1 in human prostate cell lines and attempt to distinguish contexts in which ETV1 may cooperate with or, alternatively, antagonize AR.

Remarkably, in *Pten*$^{+/-}$ mice with more evident and advanced disease, androgen regulation of target genes is no longer antagonized in the presence of the ETV1-Tg (Fig. 7), unlike in the *ETV1-Tg*$^{+}$/*Pten*^{wt} mice (Fig. 6). For the representative androgen-regulated genes *Nkx3-1* and *Clu*, expression levels between transgenics and non-transgenics differ in *Pten*^{wt} but not in *Pten*$^{+/-}$ mice (Fig. 7). Following castration *Nkx3-1* shows the expected reduction in both *Pten*^{wt} and *Pten*$^{+/-}$ mice, but in *Pten*$^{+/-}$ mice the *Clu* gene now shows a slight reduction in expression after castration rather than the expected increase, indicating dysregulation of androgen signaling in *Pten*$^{+/-}$ mice. Additionally, the overall androgen-regulated expression pattern is slightly attenuated in *Pten*$^{+/-}$ mice, for example *Nkx3-1* expression is lower and *Clu* expression is higher in intact *Pten*$^{+/-}$ mice relative to *Pten*^{wt}. The differing expression patterns may be due to an effect of the PI3K/AKT pathway on AR signaling, or may be due to strain differences (FVB/C57BL/6 F1 hybrid vs. FVB) or differences in age of the mice (24 weeks vs. mean 43 weeks). Ongoing work involves RNA-seq analysis of tumors and normal prostates from *Pten*$^{+/-}$ mice, as well as qRT-PCR analysis of RNA extracted specifically from PIN/ADC foci of FFPE slides to better gauge gene expression changes at each stage of disease progression. How PTEN loss abrogates ETV1 antagonism of AR gene regulation is currently being investigated in the lab *in vitro* in human prostate cell lines.

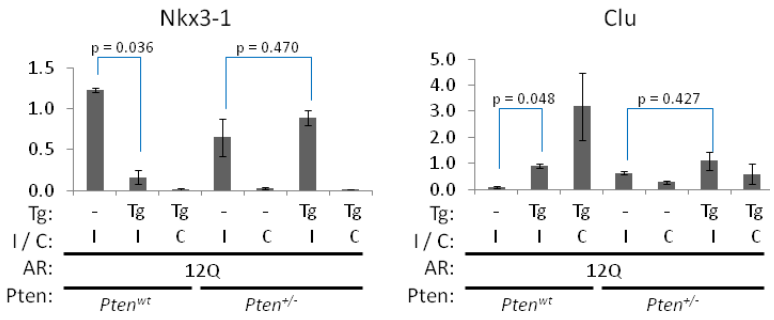

Fig. 7 - Androgen-regulated gene expression in *Pten*$^{+/-}$ mice. Genes previously dysregulated in *ETV1-Tg*$^{+}$ vs. *wt* mice no longer respond to the transgene on a *Pten*$^{+/-}$ background. The different AR genotypes show a similar pattern, so for simplicity only *hAR*^{12Q} mice are shown here as representative data. *Nkx3-1* and *Clu* are representative genes displaying antagonism of normal androgen regulation by the ETV1-Tg in *Pten*^{wt} mice but not *Pten*$^{+/-}$ mice.

Task 4. Determine human pathways signifying differential therapy response by Molecular Concept Mapping

To compare gene expression patterns in mice to the corresponding human pathways, genes identified as differentially expressed in *ETV1-Tg*$^{+}$ and castrated mice by RNA-seq were converted to human gene IDs and uploaded to the Oncomine database. The lists of genes

downregulated by the ETV1-Tg and by castration were set as "custom concepts" to be queried against existing human cancer data sets in Oncomine to find significant overlap. Molecular Concept Map analysis (1) using Oncomine data revealed that the set of genes down-regulated in *ETV1-Tg*$^{+}$ mice is significantly enriched for genes that are induced by androgens and dysregulated after androgen ablation or treatment with AR antagonists in human PCa patients and human prostate cell lines (Fig. 8). There is also significant overlap with genes altered by androgen ablation in our mice (Fig. 8). This overall pattern suggests widespread AR antagonism by the ETV1-Tg, as suspected from analysis of individual AR target genes.

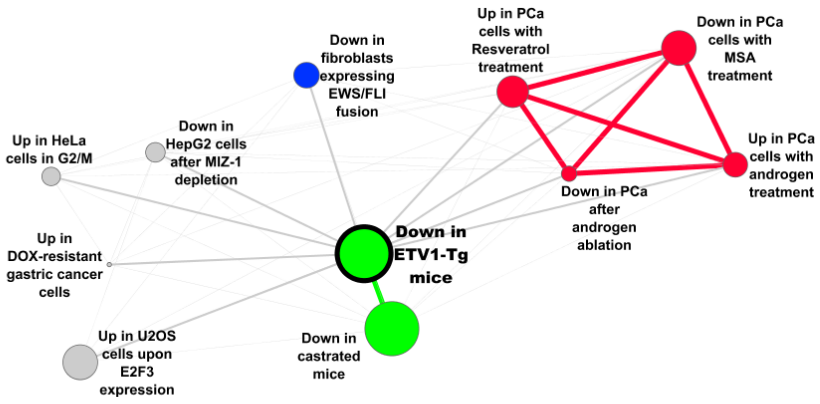

Fig. 8 - Molecular Concept Map analysis. The list of genes downregulated in *ETV1-Tg*$^{+}$ mice was uploaded to Oncomine as a custom concept ("Down in ETV1-Tg mice") and searched against the public database. The enriched gene set data was used to create a Molecular Concept Map (1) in Cytoscape. Multiple androgen-regulated gene sets (red) show significant overlap with our custom concepts (green), as does a set of genes downregulated by FLI, another ETS family member (blue).

To directly address the interaction of ETV1, AR and PTEN in a human prostate cancer model, RWPE-1 (RWPE) cells are currently being utilized. Antagonism of AR target gene expression occurred in the *ETV1-Tg*$^{+}$ mouse model of early stage disease, and RWPE cells are normal human prostate epithelial cells that have been immortalized but not fully transformed (29). RWPE cells lack endogenous AR expression, but are androgen-responsive upon introduction of exogenous AR (Fig. 9). RWPE cells stably overexpressing ETV1, ERG or LACZ (RWPE-ETV1, RWPE-ERG, and RWPE-LACZ, respectively) were a kind gift from Dr. Arul Chinnaiyan (University of Michigan). Overexpression of the ETS family member ERG, known to antagonize AR transactivation of many targets, was used as a positive control, and overexpression of LACZ acted as a negative control. We stably transduced the cells listed above with either AR or empty (FG9) expression vectors for a total of 6 experimental cell lines. To ask whether ETV1 or ERG overexpression directly alters AR activity compared to control RWPE cells overexpressing LACZ, stable cells were transfected with androgen-responsive luciferase reporters and treated with the synthetic androgen R1881, and reporter induction was measured. A luciferase reporter driven by the androgen-responsive PSA promoter/enhancer (PSA-Luc) (30) was induced by R1881 only in AR-expressing cells, confirming that the signal is AR-specific. PSA-Luc induction was significantly attenuated in RWPE-ERG cells, as predicted (26), and trended towards attenuation in RWPE-ETV1 cells (Fig. 9). A luciferase reporter driven by synthetic hormone response elements (HRE3-Luc) (31) showed induction by R1881 only in AR-expressing cells as well, with significant attenuation in RWPE-ETV1 cells (Fig. 9). Future work will determine the manner in which AR interacts with ETV1 or ERG at these and other regulatory elements, and will look at the effects on endogenous gene expression.

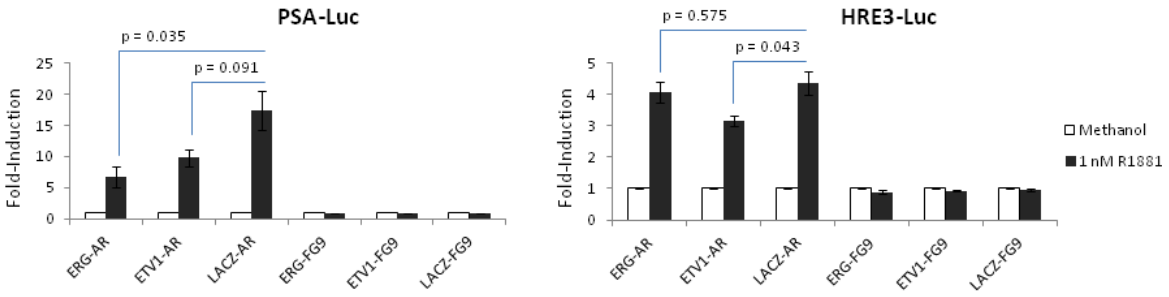

Fig. 9 - Androgen-responsive luciferase reporters in RWPE cells. RWPE cells stably overexpressing ETV1, ERG or LACZ in combination with AR or empty vector (FG9) as indicated on the y-axis were transfected with PSA-Luc or HRE3-Luc reporters and treated with 1nM R1881 or vehicle (methanol). Bars represent mean fold-induction for each cell line (relative to methanol-only treatment) +/- SEM. R1881 treatment induced reporter expression in all AR-expressing cells. ERG overexpression significantly attenuated PSA-Luc induction relative to LACZ overexpression. ETV1 overexpression trended toward attenuation of PSA-Luc and significantly attenuated HRE3-Luc induction.

KEY RESEARCH ACCOMPLISHMENTS

- A novel mouse model of PCa was developed, relying on PTEN heterozygosity, a prostate-specific ETV1 transgene, and polymorphism of AR to modulate the androgen axis

- Some of the Q-tract effect on ligand-independent AR signaling may be due to phosphorylation of AR at Ser650, correlating inversely with the length of the Q-tract and enhancing transcription at low ligand to a greater extent for short Q-tract ARs.

- *Pten*$^{+/-}$ and *ETV1-Tg*$^{+}$*/Pten*$^{+/-}$ mice develop PIN at a much higher rate than conferred by the *ETV1* transgene alone. Progression to adenocarcinoma is influenced by the presence of the ETV1-Tg and by the AR allele (effect of the latter is not demonstrable with the ETV1-Tg in a PTEN wt background).

- Androgen ablation by castration was largely preventive of disease in the *Pten*$^{+/-}$ model, although a low rate of hormone-refractory PIN and cancer was observed in castrated mice.

- *ETV1-Tg*$^{+}$ mice show reduced expression of many AR target genes relative to *wt* mice, with little effect from AR allele. The effect from the ETV1-Tg is attenuated in *Pten*$^{+/-}$ mice, with expression levels of AR targets similar between transgenics and non-transgenics. However, some AR target genes in *Pten*$^{+/-}$ mice show the opposite expression pattern following androgen ablation, and slightly reduced expression in intact animals.

- Preliminary study of AR/ETV1 interaction in human prostate cell lines supports inhibition of AR transactivation of target genes by ETV1, with a likely context-dependent effect. Further study of the mechanism of AR/ETV1/PTEN interaction is ongoing.

REPORTABLE OUTCOMES

Manuscripts resulting from this research (including a book chapter):

1) Robins DM: The Role of the Androgen Receptor Polyglutamine Tract in Prostate Cancer: In Mice and Men. In: Tindall D, Mohler J, editors. Androgen Action in Prostate Cancer. New York: Springer; 2009. p. 269-95.

2) Simanainen U, Brogley M, Gao YR, Jimenez M, Harwood DT, Handelsman DJ, Robins DM: Length of the human androgen receptor glutamine tract determines androgen sensitivity in vivo. Mol Cell Endocrinol. 2011 Aug 6;342(1-2):81-6.

3) Robins DM: Androgen receptor gene polymorphisms and alterations in prostate cancer: of humanized mice and men. Mol Cell Endocrinol. 2012 Apr 16;352(1-2):26-33. Invited review for special issue on androgen signaling.

4) Grivas PD, Robins DM, Hussain M: Predicting response to hormonal therapy and survival in men with metastatic prostate cancer. Critical Reviews in Oncology/Hematology 2012 (*in press).*

Abstracts resulting from this research:

Simanainen U, Das D, Brogley M, Gao YR, Jimenez M, Harwood DT, Handelsman DJ, Robins DM: Human AR Q-tract Length Determines *In Vivo* Androgen Sensitivity in Mice. Endocrine Society 2010, 92 (*selected for oral presentation)*

Robins DM, Starnes E, Higgins J, Brogley M: Differential Response to Androgen Depletion by Androgen Receptor Glutamine Tract Length Variation. IMPACT Meeting, 03/09/2011, Orlando, FL; poster presentation.

Seminar presentations including results from this research:

Roswell Park Cancer Institute, Verne Chapman Memorial Lecture, Buffalo, NY (07/02/08)

Department of Pharmacology, University of Minnesota, Minneapolis, MN (10/02/08)

Department of Molecular & Cellular Pharmacology, University of Miami, Miami, FL (06/24/09)

Division of Reproductive Sciences, UT Southwestern Medical School, Dallas, TX (04/12/11)

Department of Urology, University of Pittsburgh, Pittsburgh, PA (03/05/12)

Department of Cancer Biology, MD Anderson Cancer Center, Houston, TX (05/15/12)

Conference presentations including results from this research:

Invited lecture for Women in Andrology at the Andrology Society Symposium, Philadelphia, 04/05/09; *Androgen Receptor Variation and Prostate Cancer in Humanized AR Mice*.

Plenary address at the International Conference on Hormonal Steroids and Hormones & Cancer, Edinburgh, Scotland, 09/22/2010; *Genetic Variation of the Androgen Receptor in Prostate Cancer.*

Plenary address at the Endocrine Society Symposium, Boston, MA, 06/06/2011; *Genetic Variation of the Androgen Receptor: from Gene Regulation to Prostate Cancer.*

Based in part on work supported here, I have been invited to speak at upcoming meetings:
1) the Biennial Androgens Meeting, Helsinki, Finland, 11/08/12
2) the Society for Basic Urologic Research Annual Fall Meeting, Miami, FL, 11/16/12

Funding applied for based on this work:

Based on work supported here, we obtained a small pilot award from the University of Michigan Center for Genomics in Health and Medicine to perform gene expression profiling of the Q tract variant mice with ETV1 transgenes (in a wild type PTEN background), to compare quality and efficiency of microarray to RNA-seq analysis. Some of this work is discussed above and will enhance our future analysis of the PTEN mice.

Mouse strains and bioinformatic databases will be made publicly available once reported.

CONCLUSIONS

In this DOD IDEA award, we have constructed new mouse models of PCa progression relying on ETV1 overexpression, PTEN deficiency, AR polymorphism and androgen ablation. ETV1 overexpression and PTEN deficiency are both common events in human PCa and demonstrate synergy in disease progression in the mice described here. Further investigation of the prognostic value of co-occurrence of these lesions in human PCa is warranted. Additionally, disease progression in *Pten*$^{+/-}$ mice appears to be modulated by the strength of AR reflected in Q-tract differences. This observation supports previous work showing that Q-tract differences may cause differential PCa progression and treatment response, though the pattern appears to vary by model.

To examine the molecular mechanisms underlying differential Q-tract effects, we have performed cell-based assays. ARs with different Q-tract lengths are sensitive to promoter and cell type differences, and are differentially sensitive to growth factor activation that may drive AR following androgen ablation. Subtle differences conferred by Q-tract lengths may prove to be one of many factors that sum to significant differences in response to therapy.

AR is required for normal prostate development and PCa progression, hence the use of androgen ablation therapy to reduce AR signaling in human PCa. In the mouse model described here the rate of PIN/ADC drops dramatically with androgen ablation by castration, as expected, with an effect that is likely preventive rather than curative, and some hormone-refractory disease does occur. However, more *selective* antagonism of AR signaling by ETV1 overexpression or PTEN deficiency is associated with tumorigenesis. The identification of the tumor suppressor gene *Nkx3-1* as one of the AR targets downregulated by ETV1 supports the notion that antagonism of a subset of the normal AR-regulated gene expression program is an important mechanism of PCa progression, and suggests that antagonism at the molecular level may lead to synergy at the disease level. Ongoing work in the lab seeks to dissect the molecular mechanism of AR/ETV1/PTEN interaction, and further identify specific key factors in PCa progression.

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APPENDICES

Bibliography and Personnel List

Publications appended (full citation on next page):

1. Robins, chapter in Androgen Action in Prostate Cancer
2. Simanainen et al., Mol Cell Endo
3. Robins, Mol Cell Endo
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Personnel receiving pay from the research effort:

Diane M. Robins (PI) (entire period)

Michele Brogley (Res. Assoc.) (entire period)

Dweepanita Das (Postdoc) (June ’08 – July ‘09)

Elizabeth LaPensee (Postdoc) (May - June ’10, then on a training grant followed by a DOD fellowship)

Christopher Krebs (Res. Investigator) (July ’11 – April ’12)

The Role of the Androgen Receptor Polyglutamine Tract in Prostate Cancer: In Mice and Men

Diane M. Robins

Abstract The androgen receptor (AR) is critical in the initiation and progression of prostate cancer, and therefore may contribute to disease through its genetic variation. Particular scrutiny has focused on a polymorphic N-terminal glutamine (Q) tract (CAG repeat) that shows population heterogeneity. Abnormal expansion of this tract underlies late-onset neurodegeneration, and in vitro the length correlates inversely with transcriptional activity. Yet the question of whether length variation within the range of normal human alleles affects cancer has produced discordant epidemiological results, in part due to interacting genetic and environmental factors in human disease. To test Q tract length effects, the mouse AR gene was converted to the human sequence (*h*/*mAr*), creating alleles with 12, 21, or 48 CAG repeats. These mice were grossly normal, but molecular analysis revealed allele-dependent differences in target gene expression. Further, when crossed with mice transgenic for a prostate-directed oncogene (TRAMP), Q tract length-dependent differences in cancer initiation and progression were evident. TRAMP mice with short Q tract ARs exhibited earlier but more slowly progressing disease than mice with median or long Q tract ARs. Q tract length also affected disease progression after castration, but in directions opposite to those in intact mice – the AR12Q allele delayed tumor detection whereas mice with the AR48Q allele fared worse. These experiments provided evidence for a causal relationship between a human polymorphism and a cancer phenotype. In man, Q tract length effects may only be significant at extremes of variation within the normal range and may vary with stage of disease. The h/mAR mice provide an experimental paradigm in which to dissect mechanisms by which Q tract length affects development and progression of prostate cancer. Some of these mechanisms may lead to better predictors of response to therapy and new treatments targeted to the human AR.

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1 Introduction

Androgen signaling through its nuclear receptor (androgen receptor, AR) controls male differentiation, including the development and function of the prostate gland. Because of AR's regulatory role in normal growth and homeostasis, it is also a pivotal component in diseases of the prostate. Prostate cancer in particular is a substantial health problem, with an etiology and course affected by many disparate factors. Risk of disease varies with population and with environment, and many cancers remain indolent while others progress rapidly to a lethal state. Underlying this heterogeneity in disease origin and trajectory is a nearly universal dependence on androgen in early stages of tumor growth and, despite therapeutic hormone depletion, on AR in later stages. Therefore, genetic variation in AR structure and expression may impact initiation and progression of disease. This chapter focuses on a highly polymorphic region of the AR, the polyglutamine tract (polyQ, Q tract, CAG repeat). Variable length of the CAG repeat first was noted in association with neurological disease and subsequently was suspected to be a factor in prostate cancer. Epidemiological studies, however, with different human populations and different patient criteria have produced discordant results. Dissection of glutamine tract effects in vitro has provided mechanisms underlying AR activity differences, but it is unclear whether these differences impact the function of AR during tumorigenesis. To address Q tract variation in vivo and to avoid the confounding genetic and environmental heterogeneity of man, a mouse model with variant human *Ar* alleles was created that represented extremes of Q tract length that were within the normal range. The mice demonstrated Q tract length effects in prostate cancer that varied with tumor stage and with strength of the androgen axis, which reflected well the complexity of human disease. Analysis of the Q tract in mice has lended support to a broader view of AR's role in oncogenesis, which includes dictating context-dependent and opposing functions.

2 AR and the Polymorphic Polyglutamine Tract

As a member of the nuclear receptor superfamily, AR consists of a highly conserved central DNA-binding domain (DBD), a moderately conserved C-terminal ligand-binding domain (LBD), and a variable N-terminal transactivation domain (NTD). The NTD of AR comprises over half of the encoded protein and bears little similarity to functionally homologous regions of other steroid receptors. Hormone binding to the LBD alters the stable association of AR with a cytoplasmic chaperone complex to a dynamic interaction that permits nuclear localization (Pratt et al. 2004). In the nucleus the DBD recognizes response elements in target genes. The NTD participates in recruiting diverse cofactors that orchestrate transcription, in part dependent on direct contact with the hormone-dependent activation function (AF-2) in the C-terminus (He and Wilson 2002). This N/C interaction is critical for

optimal ligand binding and transactivation, and integrates differential effects of coactivators, chaperones, and post-translational modifications by ubiquitin ligases and kinases (Shen and Coetzee 2005). Promoter context-dependent interactions of the NTD with other DNA-binding proteins also enhance selectivity of AR for its targets (Robins 2004).

Significant insight into nuclear receptor physiology is possible because the AR gene is located on the X-chromosome (*Ar*; Xp11-12). Since males are hemizygous for AR, mutations are phenotypically evident, and cases of partial to complete androgen insensitivity (AIS) have revealed structure/function correlations that are informative to the steroid receptor family in general. While mutations leading to androgen insensitivity mostly occur in the DBD and LBD (McPhaul 2002), abnormal expansion of an N-terminal CAG repeat encoding a polyglutamine tract underlies the androgen-dependent late-onset neurodegenerative disease, spinal and bulbar muscular atrophy (SBMA, or Kennedy disease) (La Spada et al. 1991). The Q tract is highly polymorphic, ranging from 9 to 37 repeats in the normal population, but occurrence of 40 or more glutamine residues leads to neurodegeneration. In contrast to the loss of AR function found in AIS, Kennedy disease, like other trinucleotide repeat expansion disorders, results from both a toxic gain of function due to misfolding and aggregation of the mutant protein, as well as a partial loss of normal protein activity (Lieberman and Robins 2008). Studies in vitro ascribe the loss of function to an inverse correlation between length of the Q tract and transcriptional strength of the AR (Mhatre et al. 1993; Chamberlain et al. 1994). Given the wide range in Q tract length in the population, whether its variation underlies other androgen-dependent pathologies, such as prostate cancer, should be investigated.

In most individuals, the AR Q tract consists of between 15 and 30 contiguous glutamines from amino acid 58, with 21 CAG repeats in the reference human *AR* sequence (Zitzmann and Nieschlag 2003) (Fig. 1). In addition to the Q tract, there is a polymorphic polyglycine tract (G tract, GGN repeat) at amino acid 449, with 14–27 repeats. These trimeric repeats vary among ethnic populations in the United States, calling attention to them as possible genetic risk factors (Edwards et al. 1992). In an initial survey, the frequency of AR alleles with fewer than the median 22 CAGs was about 50% for Caucasians but much greater for African Americans and much less for Asian Americans, which parallels their differential risk of prostate cancer. These epidemiological observations suggest the hypothesis that the Q tract might be associated with prostate cancer risk, with higher risk deriving from greater activity of a shorter Q tract AR (Coetzee and Ross 1994). This was initially corroborated by finding a prevalence of short AR-CAG alleles in prostate cancer patients, especially those with advanced disease (Irvine et al. 1995), as well as somatic shortening of the CAG repeat in some tumors (Schoenberg et al. 1994). These findings are potentially important for risk assessment and prognosis, but subsequent research has produced conflicting results. Addressing the complexity requires first establishing that Q tract length differences in the normal range affect AR activity.

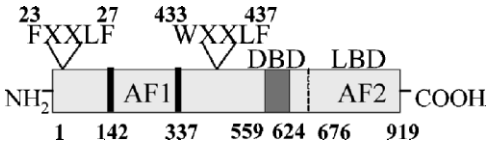

Fig. 1 Polyamino acid tracts within human, mouse, and dog ARs. The diagram shows the receptor domains for transactivation, DNA binding (DBD), and ligand binding (LBD); the former shares 85% amino acid identity between man and mouse whereas the DBD and LBD are identical, except for the intervening hinge (h) domain. The region of coding sequence that was swapped to humanize the mouse AR was stippled, and includes the polyglutamine tracts (Q_I and Q_{III}) and the polyglycine tract (G). Key amino acid positions are below the diagram for scale. Range in number of codon repeats within the polyamino acid tracts in normal populations is shown for the three species

3 Functional Analysis of the Glutamine Tract

Glutamine-rich regions are functionally important in several transcription factors besides AR, including Sp1, TATA-binding protein, and the glucocorticoid receptor (Gerber et al. 1994). These domains form β-sheets that act as polar zippers to enhance affinity with other proteins in a nonspecific manner, or, for expanded Q tracts, to promote self-aggregation (Perutz et al. 1994). More recent biophysical studies of full-length ARs revealed that the relatively disordered NTD shows Q tract length-dependent differences in secondary structure, with longer tracts conferring greater flexibility (Duff et al. 2006). Functional subdomains within the NTD encompassing distinct transactivation surfaces (e.g., TAU1, TAU5) and the N/C interaction motifs (^{23}FxxLF, ^{435}WxxLF) may be differentially masked or exposed dependent on hormone-binding, post-translational modification, and cell-specific coregulators (Dehm and Tindall 2007). The Q tract may modulate these intra- and intermolecular interactions in a length-dependent manner by functioning as a spacer between domains.

Initial mechanistic studies ascribed the reduced activity of long Q-tract ARs in transfection assays to decreased AR expression at both the mRNA and protein levels (Choong and Wilson 1998). Trinucleotide repeats are inherently unstable in DNA, and CAG repeats can form stem-loop structures in RNA that could be the target of RNA-binding proteins. Altered affinity of RNA-binding proteins due to CAG repeat length could impact both mRNA stability and translation efficiency. Precedent exists for this mechanism although such binding proteins have yet to be identified for AR. In the gene responsible for myotonic dystrophy (Yeap et al. 2004), an expanded CUG repeat in the 3′ untranslated region of the mRNA was

bound by a protein (CUGBP1) that caused aberrant RNA processing, which contributes to neurotoxicity (Mankodi et al. 2002). For AR, gene expression is also subject to complex cell-type dependent regulatory mechanisms that may be influenced by Q tract length. Furthermore, while decreased mRNA and protein levels may contribute to reduced activity of expanded Q tract ARs, significant differences in expression levels have not been noted for CAG repeat lengths within the normal range.

The first AR-associated protein found to be sensitive to Q tract length differences was Ran/ARA24, which showed decreased interaction and activation of ARs with expanded Q tracts (Hsiao et al. 1999). While RAN/ARA24 behaved as an AR coactivator in transfection assays, it is better known for its roles in cell cycle progression, maintenance of nuclear structure, and nuclear transit of RNA and protein. Differential AR activity impacted by Q tract length thus may be indirectly linked via efficiency of nuclear localization or nuclear retention. However, RAN/ARA24 has yet to show sensitivity to repeat length differences within the range of normal alleles. Such sensitivity, however, has been found for p160 coactivators. The effect is more likely due to Q tract length effects on AR's structure than to direct interaction of the tract with other proteins (Irvine et al. 2000; Wang et al. 2004). In experiments where protein expression levels were controlled carefully, AR transactivation decreased linearly with increasing tract lengths by 20% over a range of 9Q to 42Q, and more precipitously by 80% for 50Q. The presence of p160 coactivators accentuated these modest differences. Furthermore, the short tract 9Q AR responded in transfection assays to lower concentrations of androgens than moderate or long tract ARs, primarily due to enhanced N/C interaction (Wang et al. 2004). Short tract ARs increased association with coactivators as well as components of the SWI/SNF chromatin remodeling complex, which increased transactivation and androgen-dependent cell proliferation in prostate cancer cell lines. Thus modest Q tract effects caused by several molecular mechanisms ultimately combine to impact transcriptional output.

The effect of Q tract length on transactivation is not a simple linear relationship (Buchanan et al. 2004; Ding et al. 2004). AR N/C interaction seems to be optimally maintained over a critical size range of 16–29Qs. This range encompasses more than 90% of AR alleles in most populations, which suggests that pathologies may be associated with lengths at the extremes of the normal range rather than with subtle variations. This is supported by analysis of a somatic AR mutation where two nonconsecutive leucines disrupt the Q tract, presumably reducing flexibility of the NTD (Buchanan et al. 2004). For this AR-polyQ2L mutant, both protein levels and N/C interaction were reduced, but transactivation activity was increased. Thus very short or long Q tracts disrupt N/C interaction, which destabilizes AR and likely underlies reduced protein levels. This N/C interaction effect is distinct from the incremental effect of Q tract length on transactivation, for which each additional glutamine residue within the normal range may reduce structural order of the NTD, which results in reduced ability to recruit coactivators and other components of the transcriptional machinery. In contrast, interaction with corepressors appears less sensitive to differences in Q tract length (Buchanan et al. 2004). These studies

support the notion that the Q tract does not possess an intrinsic function but instead enables contact between NTD motifs and AF-2 to position AF-1 for interaction with coregulators. How the multiple, alternative surfaces within AF-1 vary in their interactions dependent on Q tract length remains to be determined.

4 Epidemiology of AR Glutamine Tract Associations

Prostate cancer has a significant genetic component, with twin studies suggesting that over 40% of cases are influenced by heredity, but whether this is due to common genetic variants or rare disease genes is unclear (Lichtenstein et al. 2000). Since AR is intimately involved in initiation and progression of prostate cancer, *AR* variation is a suspected risk factor. Although Q tract length differences within the range of normal alleles lead to demonstrable effects in vitro, studies to ascertain whether such variation impacts human physiology or cancer risk are difficult. Substantial evidence suggests that male fertility is impaired for longer Q tract lengths short of the neuropathological range (Casella et al. 2001; Davis-Dao et al. 2007). However, this association is less evident in European populations, which underscores a role for environmental factors (Yong et al. 2003). In hypogonadal men, such as those with Klinefelter's syndrome, low androgen levels fail to saturate receptors, and therefore differences in AR activity may be more apparent (Zitzmann et al. 2004; Crabbe et al. 2007). In fact hypogonadal men with short CAG repeats were more responsive to testosterone replacement, which provides a physiological correlate to the hypersensitivity of short tract ARs in vitro (Wang et al. 2004). These syndromes provide support for the notion that Q tract length differences within the normal range affect AR activity in vivo.

The hypothesis that short Q tract ARs increase prostate cancer risk was suggested by (1) the greater prevalence of these tracts in the high-incidence African-American population, (2) identification of Q tract contraction in malignant but not benign prostate cells (Alvarado et al. 2005), and (3) in vitro evidence for greater transcriptional activity of short Q tract ARs, which might enhance oncogenic transformation. But association at the genetic level has been difficult to confirm for a variety of reasons. Out of nearly 100 studies examining Q tract length and prostate cancer, about half have found an association between short tract length and increased risk, earlier age of diagnosis or more advanced disease at diagnosis (e.g., Irvine et al. 1995; Giovannucci et al. 1997; Stanford et al. 1997). However, many studies, including some with large numbers of subjects, found no association (Zeegers et al. 2004; Freedman et al. 2005), and others found the opposite association of increased risk with long Q tract lengths (Edwards et al. 1999; Suzuki et al. 2002; Li et al. 2003; Lindstrom et al. 2006a, b). The G tract has not been examined as extensively, but there is a similar level of disagreement as to whether its variation is associated with risk of prostate cancer (e.g., Irvine et al. 1995; Stanford

Au1

et al. 1997; Hsing et al. 2000; Chang et al. 2002; Zeegers et al. 2004). With regard to later stage disease and AR function, results also disagree on effects of CAG repeat length on response to androgen-deprivation treatment (Bratt et al. 1999; Suzuki et al. 2003).

These conflicting conclusions are due to several factors. Smaller studies lack sufficient power to achieve statistical significance. The association with race is subject to differences in environment, socioeconomic status, admixture, and disparity in health care. Furthermore, while the median CAG repeat number is 19 in African Americans and 22 in Caucasian Americans (Edwards et al. 1992; Irvine et al. 1995), relatively few African Americans have tracts less than the 16 Qs that are the cut-off for transcriptional effects in vitro (Buchanan et al. 2004). Heterogeneity in both genetics and environment influences outcome, as evident in opposite associations found between Q tract length and prostate cancer risk in Swedish compared to Japanese populations (Li et al. 2003). Heterogeneity in prostate cancer progression complicates results, since some studies find associations with more advanced disease or earlier age at diagnosis but no association with overall risk (Hardy et al. 1996; Beilin et al. 2001; Cude et al. 2002; Santos et al. 2003; Shimbo et al. 2005; Sieh et al. 2006). Importantly, stronger associations are found in studies with populations either prior to or less reliant on PSA testing, since these patients present with more advanced tumors. Subsequent to PSA testing, malignancies that might never become symptomatic are diagnosed at greater frequency, increasing bias to the null hypothesis (Giovannucci 2002).

Advances in genotyping technology have increased the number of genetic association studies for complex diseases such as prostate cancer, but have not resolved an association with Q tract length. A meta-analysis performed in 2004 detected a slightly increased risk associated with short CAG repeats, and this is probably an underestimation due to the inclusion of low-incidence populations (Zeegers et al. 2004). More recent studies focusing on the high-risk Swedish population found association of short Q tracts with advanced disease but disagreed with respect to overall risk. This may stem from differences in cut-off points – the study with a negative outcome defined short as $\leq$22 and long as $>$22 CAG repeats (Lindstrom et al. 2006a, b), whereas division of lengths by tertiles resolved a higher risk associated only with tracts $\leq$19 Qs (Andersson et al. 2006). Given the complexity of the epidemiological data, AR may be best viewed as a quantitative trait, the effect of which depends on variation at other loci that also vary in populations and are influenced by environment. Thus more compelling data include other genes in the androgen axis, including those encoding enzymes of testosterone synthesis, most notably *cytochrome P450* (*cyp17*), and the enzyme that converts testosterone to the more active dihydrotestosterone, *steroid-5-α-reductase type 2* (*SRD5A2*). Examining *AR*, *cyp17,* and *SRD5A2* alleles together revealed haplotypes with a significant two-fold greater risk overall (Lindstrom et al. 2006a, b). This report underscores the multigenic nature of the androgen axis in the development of prostate cancer.

5 Comparative Biology of Glutamine Tract Variation

Insights into complex diseases are often gained from comparison to animal models. Dogs are the only species other than primates known to develop age- and hormone-dependent prostate cancer (Cunha et al. 1987). Canine prostate physiology and prostate cancer progression are similar to man, and both have polymorphic *AR* Q tracts. There are two major CAG repeats in mammalian *ARs*, with the N-terminal one (CAG-I) more extensive in primates and the C-terminal one (CAG-III, at human amino acid 193) more extensive in rodents (Choong and Wilson 1998; Lu et al. 2001) (Fig. 1). A very short CAG-II just proximal to CAG-I is less variable between species. While most species have preferentially amplified either CAG-I or CAG-II, dogs are unusual in having long tracts at both positions, although CAG-I is shorter than in man. Only primates have an extensive G tract.

Given the rich diversity of canine breeds, Q tract variation was assessed in multiple breeds and an association with prostate cancer incidence sought using data on dog prostate cancer incidence from the Veterinary Medical Data Base of Purdue University via collaboration with Dr. Vilma Yuzbasiyan-Gurkan of Michigan State Veterinary Medical Center. Of over 200,000 cases presenting for any medical problem, approximately 0.5% of male dogs had prostate cancer. In addition, incidence varied significantly among breeds. For example, 3.5% of intact male beagles presented with prostate cancer compared to only 0.5% of golden retrievers. Therefore, polymorphism in CAG-I and –III within and between breeds was analyzed for ten individuals of each of five breeds with low prostate cancer incidence (golden retriever, Siberian husky, cocker spaniel, dalmatian, and greyhound) and five breeds with high incidence (beagle, Scottish terrier, Rhodesian ridgeback, doberman pinscher, and Shetland sheepdog). Both canine Q tracts are polymorphic, with 9–12 Qs in CAG-I and 21–24 Qs in CAG-III (Shibuya et al. 1993). Two allele lengths were observed for CAG-I and 4 for CAG-III. While some variation in both tracts occurred within both groups, association of short tracts with tumorigenesis by breed did not reach statistical significance. More compelling data came from analysis of individual dogs with spontaneous disease. Of 13 histologically confirmed prostate cancer samples, ten had the short allele at CAG-I and either the shortest of the four alleles at CAG-III, or an even shorter allele absent from normal dogs. Moreover, two tumors were heterogeneous in CAG-III, displaying yet shorter alleles indicative of contraction during tumor progression. This pilot study supports the hypothesis that ARs with short Q tracts, in men or dogs, confer greater prostate cancer risk, and further, that such receptors arise and are selected for during tumorigenesis.

Mice are preferable to dogs as models of human disease pathogenesis because of smaller size, faster generation time, and the homogeneous genetic background of numerous inbred lines. Although mice have a lobular prostate and develop spontaneous prostate cancer rarely, xenograft and germline modification techniques compensate for these deficiencies. All stages of disease can be sampled, in contrast to clinical specimens in which early events are more difficult to observe. However,

the mouse Q tract differs significantly from the human (Fig. 1). Rodents have an abbreviated CAG-I and while rats have 20 Qs in CAG-III, the mouse tract is disrupted by histidines ($Q_8HQ_3HQ_2HQ_4$), likely influencing its flexibility as an interdomain spacer. Exclusive of the Q tract, the amino acid sequence of the mouse and human NTDs differ by 15%, in contrast to the DBD and LBD that are identical except for eight amino acids in the hinge region. In transfection experiments, the reduced transactivation strength of the rodent AR relative to human maps to the NTD (Chamberlain et al. 1994). Therefore, to create a mouse model to assess Q tract length effects on phenotypes and disease, genetic engineering was used to "humanize" the mouse AR, by swapping sequences encoding the human NTD into the mouse gene.

6 Development of "Humanized" AR Mice to Study Glutamine Tract Function

To convert the mouse AR to the human sequence, targeting vectors for germline homologous recombination that used a backbone of a 129/Sv mouse genomic fragment encompassing mAR exon 1, which encodes almost the entire NTD. Amino acids 31–484 (including a median length Q tract of 21 residues) from human AR cDNA were substituted for the equivalent mouse sequences via conserved restriction sites, and a neomycin selectable marker was introduced into intron 1, near the 5′ splice junction (Albertelli et al. 2006). Additional vectors were constructed to create AR alleles with 12 or 48 Qs, which represented extreme lengths within the normal variation range. These DNAs were electroporated into mouse embryonic stem (ES) cells where homologous recombination occurs via the substantial 5′ flanking and intronic chromosomal sequences in the vector. The selectable marker was excised from ES clones by transfection of a plasmid expressing cre-recombinase, which produced a transcription unit with the humanized AR under the control of mouse regulatory sequences. This "h/mAR" sequence differs from human in retaining mouse codons for one N-terminal amino acid, 14 residues just before the DBD (ten of which are conservative changes) and eight amino acids (four conservative) in the hinge region. The targeted ES cells were introduced into mouse blastocysts, and chimeric progeny were used to establish lines of h/mAR mice that have been backcrossed onto the C57BL/6 background.

Both sexes of the h/mAR mice were indistinguishable from wild-type mAR littermates in gross physiology including behavior, growth rate, body weight, and lifespan (Albertelli et al. 2006). All three strains were fertile with similar frequency of litters and number of pups per litter. In particular, while longer Q tracts were associated with reduced fertility in man, the 48Q male mice remained as fertile as the other genotypes even at older ages. A preliminary analysis of the androgen axis indicated no significant differences between genotypes in serum testosterone, LH or FSH levels, although individual values varied over a broad range. Whether the

androgen axis is sensitive to Q tract length will be tested more stringently for mice that have been backcrossed additional generations and are individually housed to eliminate effects of social interaction. When seminal vesicle weight was used as a more direct indicator of androgen action, differences among genotypes became apparent; seminal vesicle weight was lower in h/mAR48Q mice at 6 months and greater by 2 years of age in h/mAR12Q mice. These data confirm an inverse correlation between AR activity and Q tract length, and suggest that minor effects may sum over time to detectable phenotypes.

The h/mAR48Q mice were studied further since 48 CAGs may cause partial androgen insensitivity or Kennedy disease in man. However, gene defects often must be more severe in mice than man to model a human syndrome, perhaps due in part to differences in lifespan and, relevant to both trinucleotide repeats and cancer, differences in genomic instability. While transgenic ARs with 65Qs produced no phenotype in mice (Bingham et al. 1995), mice created by introducing 112Qs with our targeting vector replicated the pathology of Kennedy disease (Yu et al. 2006). The h/mAR48Q mice showed no evidence of neuromuscular deficiency, as determined by grip strength tests, even at advanced ages. Even though fertility remained normal, some variation in testis function was discernable at the molecular level. In particular, mRNA levels of *Hsd17b3*, an indicator of mature Leydig cell function, were significantly lower in 48Q than other mice, although levels were still within a normal expression range. Further, although AR protein levels were similar between genotypes, and no histological evidence of aggregation was observed in AR48Q cells as reported in Kennedy disease, this protein may be predisposed to aggregate since substantial amounts were found in the pellet fraction of whole cell lysates. Therefore, h/mAR48Q, while sufficient for male mouse differentiation and virility, may have suboptimal activity in some circumstances.

This characterization confirmed that human and mouse ARs have sufficient conservation, as might their interactions with critical coregulators, to substitute functionally for one another at the organismal level. Furthermore, while minor phenotypic variations were detected for h/mARs with 12 or 48 Qs, these were mostly within the normal range of variation for these traits and did not produce pathology. These alleles in mice may model the extremes of Q tract length within the range of normal alleles similar to those found in man, which allows investigation of whether these variants influence prostate biology or disease. At a gross level, the three h/mAR strains appeared no different from wild-type mice in prostate morphology, with equivalent epithelial and nuclear AR at the immunohistochemical level. At a molecular level, however, differences in AR regulation were detected using quantitative RT-PCR. AR mRNA levels showed a trend to inverse correlation with Q tract length in 6-month-old mice. More significantly, the variation in expression of some AR target genes was consistent with greater transcriptional activity of the 12Q allele and reduced activity of the 48Q allele (Fig. 2). In particular, androgen-regulated probasin, a protein secreted from mature prostatic epithelium, was expressed at higher levels in 12Q than 21Q or 48Q h/mAR mice. *Nkx3.1*, a prostate-specific and AR-dependent homeobox gene, showed significantly lower levels in h/mAR48Q mice. In contrast, clusterin

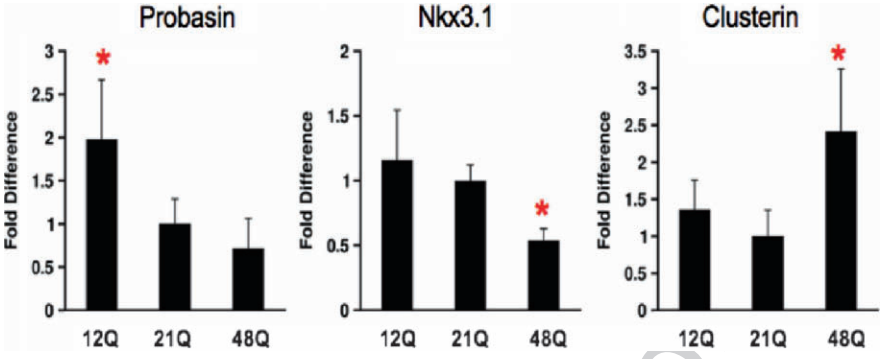

Fig. 2 Prostate gene expression was influenced by AR Q tract length. Prostate mRNAs from 3 to 8 mice at 6 months of age per h/mAR allele were quantified using real-time RT-PCR. Results are relative to levels in wild-type mAR mice. Probasin and Nkx3.1 were up-regulated by AR, clusterin was repressed. *Asterisks* indicate significant differences. Modified from Albertelli et al. (2006), *Copyright 2006, The Endocrine Society*

(an antiapoptotic protein also known as testosterone-repressed prostate message-2), which was down-regulated by AR (July et al. 2002), had higher expression in h/mAR48Q mice. Q tract length appears to impact not just AR activated but also AR-repressed genes. Overall, differences in target gene expression due to h/mAR allelic variation were small, but consistently trended to greater activity of the 12Q allele and lesser activity of the 48Q allele, for both induction and repression. These results support the hypothesis that Q tract length impacts differential transcription of critical androgen-dependent genes, some of which may impact prostate cancer risk.

A broader view of differential regulation by AR alleles was obtained from a preliminary comparison of prostate gene expression using Affymetrix oligonucleotide microarrays. Stringent statistical criteria did not reveal significant differences between AR alleles (including mAR), but when data were analyzed without adjusting for multiple comparisons, some intriguing patterns emerged (Albertelli 2007). For example, differentially expressed genes were frequently at highest levels in h/mAR48Q mice, which corroborated studies that reported more genes were repressed than activated by AR in the prostate (Desai et al. 2004). h/mAR12Q and h/mAR48Q expression often trended in the same direction relative to that of h/mAR21Q, which suggested that for many promoters the median Q tract length provides optimal activation (or repression), with deviation to either longer or shorter tracts having similar effects. Frequently only the 12Q or the 48Q differed from the other two alleles, which suggested that Q tract effects may be promoter-context dependent and encompass multiple mechanisms. Specific transcripts showing differential expression include some previously noted as either androgen-regulated or overexpressed in prostate cancer, such as several involved in inflammatory responses. Of particular interest are a few genes up-regulated in h/mAR48Q mice that are involved in Wnt signaling, especially Wnt5a, whose twofold greater expression was validated using Q-PCR. Crosstalk occurred between

AR and Wnt signaling pathways and Wnt overexpression has been associated with androgen-stimulated prostate cancer (Yang et al. 2006). In this case, higher expression of Wnt5a in h/mAR48Q mice may be a factor in their development of aggressive castration-recurrent cancer (see later). More definitive analyses using laser capture microdissection will provide additional information on genes and pathways influenced by AR Q tract length.

7 Glutamine Tract Length Effects in Mouse Prostate Cancer

h/mAR mice were crossed to the engineered model TRAMP (transgenic adenocarcinoma of the mouse prostate) to investigate the effect of Q tract length variation on oncogenesis. TRAMP mice carry the SV40 T antigen (Tag) oncogene driven by the prostate epithelium-specific probasin promoter and enhancer (Greenberg et al. 1995). TRAMP males develop prostatic intraepithelial neoplasia (PIN) and well-differentiated prostate cancer that seems histologically similar to the human disease (Kaplan-Lefko et al. 2003). PIN generally develops by 12 weeks of age, and tumors are abdominally palpable somewhat later; metastasis occurs predominantly to lymph nodes, lungs, and liver, but not bone. Development of prostate cancer in TRAMP males is fully penetrant and can be delayed by castration, but ultimately progresses, indicating that disease becomes androgen independent, as in man. While T antigen is a more potent inducer of disease than in human prostate cancer, abrogation of Rb and p53 function occurs in both. TRAMP mice have been invaluable for studying early events in prostate cancer and for evaluating treatment and prevention strategies, although more recent models that incorporate inactivation of the tumor suppressor PTEN may more accurately mimic the origins of human disease (Wang et al. 2003). Regardless of how these models induce cancer and despite the genetic uniformity of mice, there is heterogeneity in time of initiation and rate of progression, which demonstrates the stochastic nature of tumorigenic events. Some of these events are genetic, as they vary with strain background, and some may be epigenetic and subject to diet, inflammation, and hormonal parameters.

h/mAR-TRAMP mice and mAR-TRAMP littermates were compared to determine whether differences in Q tract length affected the development of PIN (Albertelli et al. 2008). PIN in mice is the precursor to carcinoma and is characterized by features including epithelial tufting, nuclear hyperchromasia, amphophilic cytoplasm, apoptotic debris, and cribriform architecture (Shappell et al. 2004). At 12 weeks of age, each prostate from ten mice per AR allele had varying levels of PIN in all prostatic lobes. The amount of dorsal epithelium associated with PIN per dorsal lobe was quantified by scoring into categories 1–10, which approximated the percentage of involvement (Fig. 3). A compelling trend was evident, although within each cohort interindividual differences precluded reaching statistical significance. More 12Q mice clustered at higher levels of PIN (70% in category 6 or above), while the majority of 48Q mice exhibited lower PIN

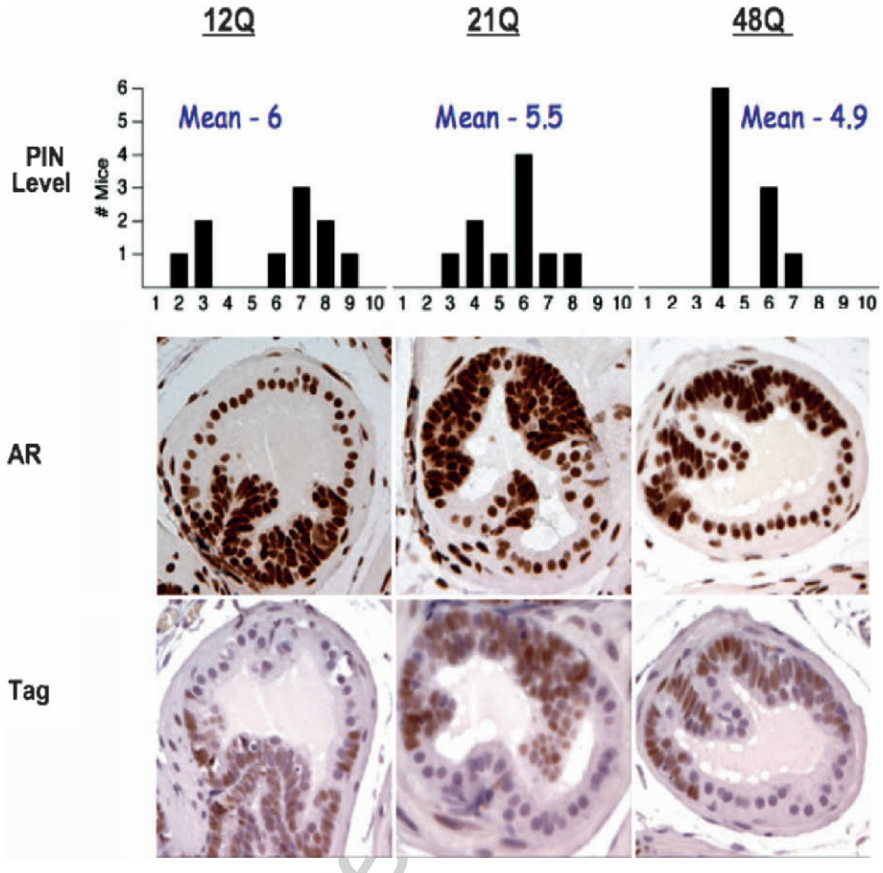

Fig. 3 The amount of PIN was inversely proportional to Q tract length. The amount of epithelium involved in PIN at 12 weeks of age was assessed in sections of dorsal lobes, ten prostates per allele, and categorized by approximate % PIN involvement (categories 1–10, where 1 represents about 10% and 10 about 100% PIN involvement). Distribution per genotype showed a trend to inverse correlation with Q tract length (median categories of 7, 6, and 4 for 12Q, 21Q, and 48Q, respectively). Areas of PIN identified by H&E staining were examined in sequential sections for AR and Tag expression using IHC. Overall, AR and Tag expression was similar among Q tract variants, with AR fairly uniform in epithelium and stroma, while Tag expression was highest in areas of PIN. Modified from Albertelli et al. (2008), *Copyright 2008, Oxford University Press*

levels (60% in category 4). Furthermore, a higher grade of PIN, distinguished by greater expansion of the gland, cribriform proliferation, and more pronounced hyperchromasia, was detected in some of the 12Q and 21Q, but not 48Q, mice. Therefore, AR Q tract length had a notable affect on PIN by 12 weeks of age, with shorter tracts promoting more and higher-grade PIN than longer tracts. The higher levels of PIN may reflect increased proliferation rates or decreased apoptosis, which would also influence subsequent oncogenesis; whether these are affected by Q tract length is under study.

The h/mAR mice expressed similar levels of prostatic AR mRNA in all genotypes, and immunohistochemistry (IHC) showed similar levels of AR protein in epithelial and stromal nuclei (Albertelli et al. 2006; Albertelli et al. 2008) (Fig. 3). AR in areas of PIN appeared slightly more intense than in normal epithelia, but this likely reflected the higher cell density in these regions. In contrast, although Tag did not show differential staining dependent on genotype, it was more prominent in areas involved in PIN than in normal epithelium. AR allele-dependent differences in Tag expression below the level of detection of IHC may exist, since expression of probasin, whose promoter drives the transgenic oncogene, varied with AR allele in the parental strains. Furthermore, functions of AR in stroma vs. epithelia may enter into higher Tag expression in regions of PIN compared to normal epithelia. Altogether, a cascade of events in neoplastic transformation may enhance Tag expression. In some regards, Tag is analogous to the translocation product TMPRSS2:ETS, in which an androgen-responsive promoter is fused to a transcriptional regulator of proliferation as a common and early event in human prostate oncogenesis (Tomlins et al. 2007). Fusions of this type may maximize the contribution of (or the sensitivity to) the androgen signaling axis in prostate cancer progression.

Tumorigenesis was followed in h/mAR-TRAMP mice by abdominal palpation, which detects tumors only a few days later on average then their identification by more costly and time-consuming magnetic resonance imaging (Albertelli et al. 2008). Tumors become palpable at approximately 1 cm in diameter, at midstage disease. Kaplan-Meier analysis revealed a significant difference among genotypes in time to tumor detection (P value <0.0001; Fig. 4), with the median age of detection 10 weeks later for 48Q mice than for the other genotypes. Tumors were initially palpable in 12Q, 21Q, and mAR mice over a similar age range (median: 25 weeks for 12Q and 27 weeks for 21Q), but the kinetics of progression differed among genotypes. Comparing disease status at 29 weeks of age, by which time about half of the 21Q and mAR mice have a detectable tumor or have already died, overt disease was detected in 85% of the 12Q mice, and in less than one-third of the 48Q mice. Age of death further distinguished the genotypes by Kaplan-Meier analysis (P value <0.0001). mAR and 21Q mice survived to mean age of 30 weeks, compared to 42 weeks for 12Q and 55 weeks for 48Q mice. Taken together, the slightly greater PIN levels at 12 weeks, more tumors detectable by 29 weeks, and longer survival relative to 21Q mice suggest that in 12 Q mice tumors initiate earlier but progress more slowly than for the other AR alleles. This is corroborated by comparing "disease length," noted as the time between initial tumor detection and death. Disease length is longer, and ranges more widely, in 12Q and 48Q mice than 21Q or mAR mice. Given the genetic homogeneity of these mice, stochastic events appear to increase heterogeneity in tumor progression as well as initiation.

End-stage tumors using IHC and a tissue microarray were used to explore whether the different AR alleles played a role in heterogeneous tumor progression. Shorter disease length corresponded to poorly differentiated or undifferentiated tumors with heterogeneous AR staining for all genotypes (Fig. 5). In contrast, the majority of 12Q and 48Q mice that had long disease lengths had well- or moderately

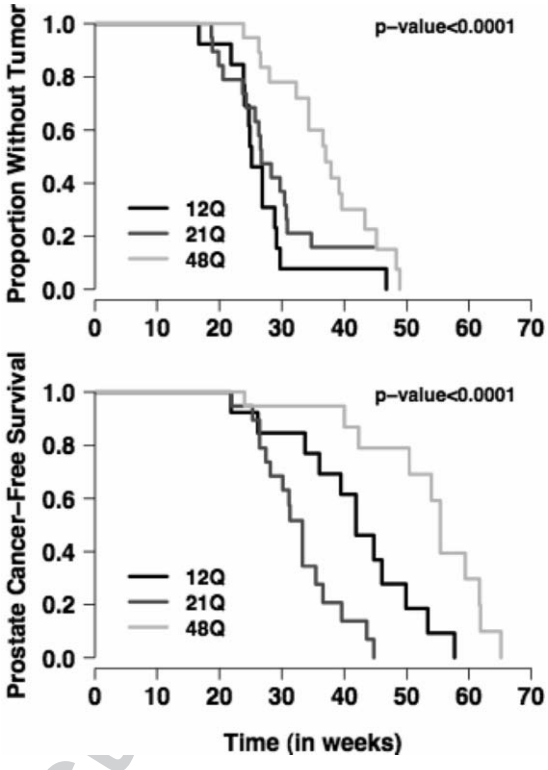

Fig. 4 Q tract length affected age of tumor detection and survival in intact mice. The upper panel shows proportion of mice without a palpable tumor and the lower panel shows prostate cancer-free survival; *P* values are from log-rank analysis. Number of mice per group (*n*) was: mAR = 18, 12Q = 13, 21Q = 19, 48Q = 19. Modified from Albertelli et al. (2008), *Copyright 2008, Oxford University Press*

differentiated tumors with high levels of AR expressed in most epithelial and stromal cells. IHC results were confirmed using Q-PCR and Western blot analysis of gross tumor samples. For all genotypes, greater differentiation and slower progression was associated with AR levels higher than benign prostate, and was influenced by Q tract length. In contrast, Tag levels were heterogeneous regardless of AR allele, level of expression, or disease length, which corroborated a general uncoupling from AR regulation that was evident in advanced tumors (Kaplan-Lefko et al. 2003).

These TRAMP tumors also differed in their extent of neuroendocrine phenotype, as indicated by synaptophysin expression. In man, neuroendocrine differentiation is associated with aggressive, castration-recurrent prostate cancer that mostly lacks AR expression (Shariff and Ather 2006). In TRAMP neuroendocrine cells are also associated with aggressive disease, but they can express AR (Kaplan-Lefko et al. 2003). Most 21Q tumors express moderate levels of both synaptophysin and AR. 48Q tumors showed the least synaptophysin expression, with only about half of the tumors positive for the marker and only half of those also showing AR expression. Tumors from 12Q mice were more similar to the human phenotype; synaptophysin and AR expression were mutually exclusive, with half of the tumors expressing synaptophysin and the other half expressing AR. Overall, synaptophysin expression in the TRAMP tumors, as in human disease, is most often associated with rapid

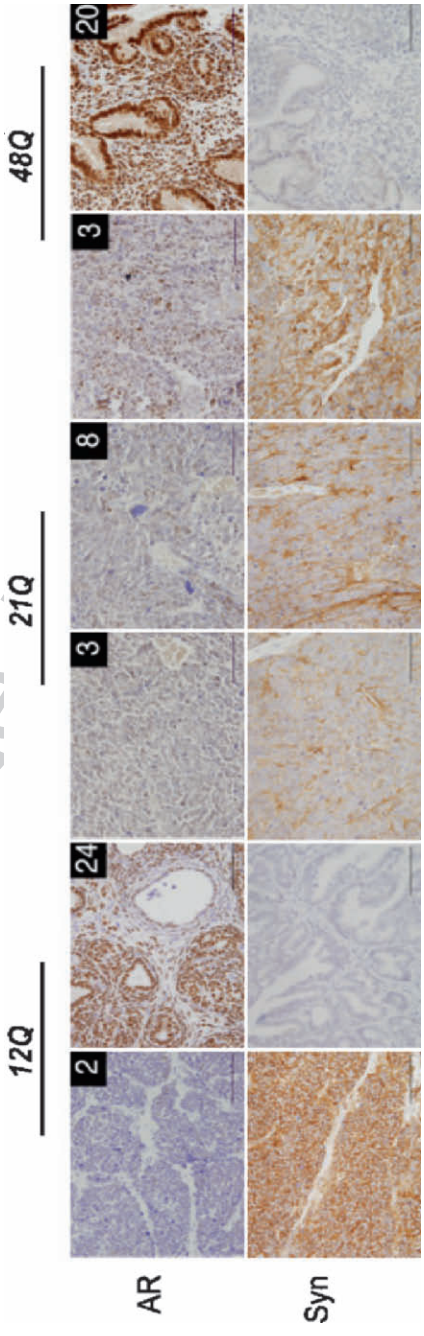

Fig. 5 Markers of prostate cancer progression in intact h/mAR-TRAMP mice. End-stage tumors were used to create a tissue microarray and sections were stained for AR (*above*) and synaptophysin (*below*) (with *vertical rows* from the same tumor). Two tumors are represented per genotype, the left one represents shorter disease length and the right one longer disease length; disease length in weeks is indicated in the black box in the upper right. 12Q and 48Q tumors of longer disease length had greater differentiation and AR expression, and less synaptophysin expression. Bar = 100 μM. Modified from Albertelli et al. (2008), *Copyright 2008, Oxford University Press*

progression. A complementary pattern was shown for expression of clusterin, an antiapoptotic protein more often associated with more differentiated tumors (Caporali et al. 2004).

Study of this mouse model of AR Q tract length polymorphism confirmed some expectations from epidemiological data. Disease initiation follows a linear trend from earlier occurrence in mice with shorter Q tracts to significant protection for longer Q tracts. Disease progression, however, follows a more complicated course. The effect on initiation likely stems from increased or earlier Tag activation by shorter Q tract ARs that hastens the time until some critical level for neoplasia is reached. Early androgen-dependent events in human prostate cancer may act similarly in the case of TMPRSS2 fusion genes (Tomlins et al. 2007). Tumors in mice with long Q tract ARs initiate later and progress slowly, perhaps modeling indolent disease more commonly found in aging men. In the 12Q mice, tumors progress along divergent paths – some show a more aggressive phenotype similar to that driven by the median Q tract AR, but most exhibit a slow-growing well-differentiated disease. Some of this apparent complexity may reflect differences in the androgen axis in man and mouse and consequences of this in neoplasia.

Since the AR Q tract length affects androgen-sensitive tumorigenesis in mice, h/mAR-TRAMP mice orchiectomized at 12 weeks of age were monitored to test whether this variation also influenced castration-recurrent cancer. Since adrenal androgen synthesis differs in mice and men, castration reduces serum testosterone to very low and perhaps undetectable levels. TRAMP tumors arise from pre-existing PIN lesions and can be compared to those in men during androgen-deprivation therapy. Remarkably, Q tract length elicited differences in tumor detection and progression in castrated mice, and in directions distinct from those in intact mice (Albertelli et al. 2008). The mice with short Q tract ARs trended to delayed tumor detection by Kaplan-Meier analysis (P value = 0.079) compared to those with median and long Q tracts, which followed a disease course very similar to each other in time to palpation and age of death (Fig. 6). This difference became apparent at 29 weeks of age when palpable tumors were present in only ~15% of 12Q mice, whereas about half of the 21Q and 48Q mice had detectable tumors or had died. For all genotypes, the time from tumor palpation until death was shorter following castration than in intact animals, but was significantly longer for castrated 12Q mice than for the other alleles. In some animals, tumors either never became palpable or remained small, and these mice died of metastatic disease found at necropsy. Remarkably, half of the 12Q mice succumb in this manner, compared to only about 10% of the 21Q and 48Q mice. Furthermore, 2 of 14 castrated 12Q mice survived beyond the term of the experiment and were euthanized at the ages of 16 months and 2 years, with no gross evidence of disease upon necropsy. Thus the mice with short Q tracts followed distinct pathways of disease, one distinguished by delayed onset of large primary tumors and the other by late onset of aggressive metastatic disease. The prevalence of metastasis as cause of death in 12Q mice is reminiscent of epidemiological data indicating aggressive disease as a common feature of men with short Q tract ARs, such as found in the African-American population (Bennett et al. 2002).

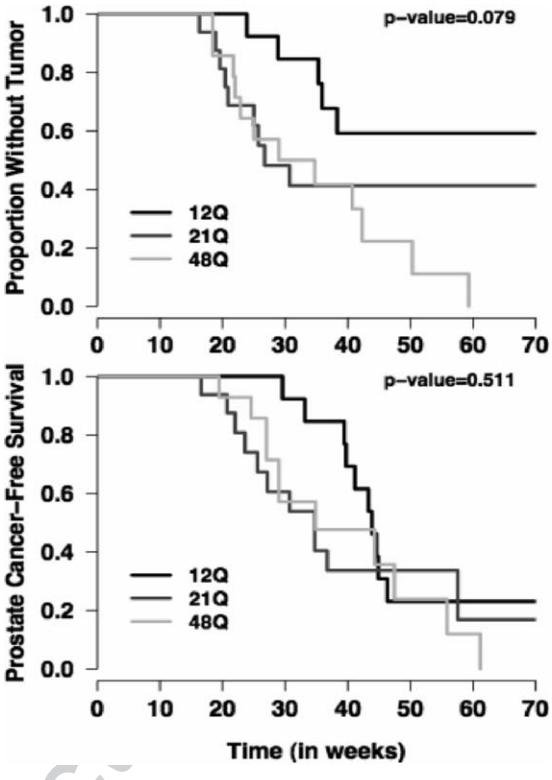

Fig. 6 Q tract length inversely affected tumor detection and survival in castrated mice. The upper panel shows proportion of mice without a palpable tumor and the lower panel shows prostate cancer-free survival; *P* values from the log-rank statistics are shown. Number of mice per group (*n*) was: 12Q = 13, 21Q = 16, 48Q = 14. Modified from Albertelli et al. (2008), *Copyright 2008, Oxford University Press*

Tumors from castrated TRAMP mice were mostly undifferentiated by histological analysis, in accord with the short survival time once tumors become palpable. AR IHC staining was modest and heterogeneous, but generally nuclear despite the presumed absence of testosterone. However, the small primary tumors of 12Q mice that died of metastatic disease were more differentiated with cytoplasmic as well as nuclear AR immunostaining that was distinctly higher overall than in the other genotypes (Fig. 7a). These small tumors also failed to express synaptophysin, in contrast to the larger palpable tumors from this group. Interestingly, the 48Q tumors also lacked synaptophysin expression, unlike 21Q tumors, even though these mice had similar ages of tumor detection and death, and similar gross histology. These phenotypic markers further illustrate that alternative courses of tumor progression are influenced by ARs with different Q tract lengths.

The effect of AR Q tract length was accentuated by comparing intact and castrated mice of the same genotype. h/mAR21Q-TRAMP mice behaved similar to mAR-TRAMP mice, with a large proportion of those castrated exhibiting delayed tumorigenesis and death, but also with a substantial number developing tumors and succumbing earlier than intact counterparts. These alternative responses to castration were polarized in the 12Q and 48Q mice (Fig. 7b). In 12Q mice, tumor detection was significantly delayed in castrated compared to intact mice (*P* value

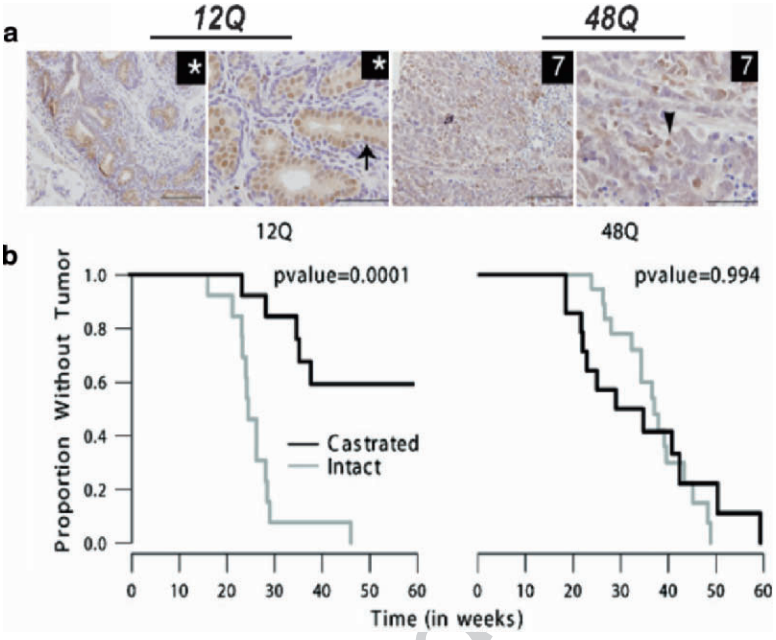

Fig. 7 Prostate cancer progression in castrated h/mAR-TRAMP mice. (**a**) Representative end-stage tumors from 12Q and 48Q castrated mice, stained for AR, at low (*left*) and high (*right*) magnification. The 12Q tumor (*) was a nonpalpable tumor from a mouse that died of metastatic disease. The 48Q tumor had a 7-week disease length. High magnification showed significant cytoplasmic AR in the 12Q tumor, and more nuclear AR in the 48Q tumor. (**b**) The response to castration varied with Q tract length. The 12Q AR shifted the balance to tumors developing later after castration, but the 48Q AR responded poorly to treatment. Modified from Albertelli et al. 2008, *Copyright 2008, Oxford University Press*

<0.0001), although an effect on survival was modest due to the long disease length of intact 12Q mice. In contrast, while 48Q mice showed little difference in time of tumor detection between intact or castrated mice, tumor progression was more rapid following castration and survival times were significantly shorter (P value = 0.011). Thus the short Q tract was associated with a positive response to earlier castration in mice. Androgen-independent cells are thought to exist in TRAMP mice prior to castration, and castration may synchronize these cells and drive selection for aggressive growth (Johnson et al. 2005; Wikstrom et al. 2005). This may be analogous to results of the finasteride prevention trial in man, in which reduced dihydrotestosterone levels decreased the number of prostate cancer cases but more of those occurring were of higher grade (Thompson et al. 2003). Since tumors arise later in 12Q mice following castration, and survival is decreased in 48Q mice by castration, AR strength may impact response to castration, with a stronger AR producing a more favorable outcome. Some studies have noted a similar effect in man, where low testosterone prior to treatment, which may suggest a weaker androgen axis, as in 48Q mice, correlated with poor prognosis (San Francisco

et al. 2006). Overall, despite differences between the human and murine disease, the 12Q and 48Q mice provide a model in which response to hormonal treatment is genetically programmed. Further analysis may reveal tumor characteristics that could serve as early biomarkers in man for predicting response to androgen-deprivation therapy and ultimately suggest distinct strategies for treatment.

8 Conclusions

Humanized AR mice with alleles varying in Q tract length provide an in vivo model to test the role of this polymorphism in androgen-dependent traits and in the etiology of disease. In creating these mice, Q tract lengths were chosen at the extremes of those found in the normal human population to optimize detection of informative differences. The resulting strains are indistinguishable from wild-type mice at a gross level, but reveal AR allele-specific variation within the normal range in some physiological and molecular indicators of androgen action. Therefore modest phenotypic alterations, directly or indirectly due to differences in transcriptional activity of the variant Q tract ARs, are detectable and may be cumulative with age. These variations are accentuated in mice by their genetic and environmental homogeneity, but also may exist in man (Zitzmann and Nieschlag 2003). Our data and those of others suggest that they may be most penetrant for alleles with CAG repeat numbers at the extremes of the normal range.

Polyglutamine-tract-dependent effects in the h/mAR mice are more pronounced in the context of cancer, with statistically significant differences in tumor detection and survival evident in the TRAMP model. The probasin promoter that drives the Tag oncogene is the likely initial oncogenic sensor of AR strength, but the critical differential gene activation may occur early or in a subset of cells since genotypic differences in Tag expression are not evident at a gross level. Moreover Tag expression is not the sole determinant of disease course, since significant differences occur in progression as well as tumor onset in these strains. Whether early hyperplasia becomes neoplasia depends not only on proliferative capacity of the epithelia cells but also on growth-promoting vs. inhibiting effects of stroma. These opposing forces are influenced overall by the androgen axis, and thus may be modulated directly and indirectly by AR Q tract effects and further exacerbated by neoplasia. Mechanisms that under homeostatic conditions limit the effects of genetic variation (e.g., AR alleles) and environmental change (e.g., hormone levels) may be overcome by the more global dysregulation accompanying oncogenesis. One such mechanism is sequestration of AR by chaperone molecules such as heat-shock protein 90. Cryptic genetic variation and subclinical phenotypes may be kept in check by chaperones, but unmasked and amplified by stress or disease states.

Cancer progression in TRAMP mice does not show a linear correlation with CAG repeat number, and short-term effects may differ from long term, complexities perhaps also underlying discordant results in man. Our studies suggest similar disease courses in intact 12Q and 48Q mice may occur through distinct mechanisms

at the cellular level, due to disparate influences of these alleles on downstream targets involved in proliferation and differentiation. Early in disease, AR maintains its normal function as a tumor suppressor, so increased transcriptional activity associated with a short Q tract slows tumor progression by favoring differentiation. Later in disease, multiple events conspire to switch AR's function to that of an oncogene and promote tumor growth (Litvinov et al. 2003). Thus reduced activity associated with the long Q tract delays tumor initiation and slows tumor progression in 48Q mice. Q tract variation is likely to affect AR activity differentially in stroma vs. epithelia due to the presence of differing amounts or types of cofactors and the array of active signaling pathways. Comparison of gene expression profiles in these two cell types and at different stages of disease may define multiple pathways and precise mechanisms by which these variant AR alleles influence androgen-dependent prostate cancer. In man, extremes within the normal range may favor divergent pathways of disease progression, although modest differences in CAG repeat number are likely insignificant.

Comparison of disease progression in castrated TRAMP mice is complicated by the androgen dependence of the transgenic oncogene's promoter. Nevertheless, striking differences highlight influences that may also operate in human disease, for instance via TMPRSS2 fusion genes. The divergent responses of the 12Q and 48Q alleles to castration suggest that an effect of Q tract length on ligand-independent AR activation may enter into castration-recurrent disease. Moreover, the slow growth and differentiated phenotype of 12Q tumors relative to the rapid progression of 48Q tumors after castration suggests some residual AR activity may be advantageous in treatment. This benefit pertains largely to the primary tumor in the TRAMP model since androgen-independent and probably AR-negative cells ultimately metastasize. Analysis of Q tract effects in clinical prostate cancer may be more difficult due to this dichotomy between slow tumor growth and aggressive metastasis. Detection of insignificant prostate cancer by PSA testing and disease heterogeneity may confound treatment effect further. These distinct pathways of progression following androgen-deprivation therapy suggest continued sensitivity to AR activity and the androgen axis. The role of AR during androgen-deprivation therapy will be clarified by examining the Q tract variant alleles in the context of an oncogenic model not directly driven by androgen, such as in mice with prostate-specific deletion of the *Pten* tumor suppressor.

In summary, the Q tract variant h/mAR alleles provide a novel genetic paradigm that allows investigators to establish a causal relationship between a polymorphism and a phenotype. Extremes of the normal range of variation produce demonstrable differences in laboratory mice in a uniform genetic and environmental context. Some of these differences may be direct transcriptional effects and some may result indirectly from systemic modulation of the androgen axis. These differences are more apparent, and more complex, in cancer, which suggests that Q tract effects vary with cell type and disease stage. As a risk factor in man, extremes of the normal allelic range may have modest predictive value that take on more significance in combination with certain alleles for androgen metabolizing enzymes. Furthermore, extremes of Q tract length may affect cancer progression, and of more clinical

relevance, response to androgen-deprivation therapy. Recent evidence from mouse models suggests ablation of AR function may not be the best strategy for prostate cancer, but rather in at least some situations may select for more aggressive AR-negative cells (Johnson et al. 2005; Wikstrom et al. 2005; Banach-Petrosky et al. 2007). Elucidation of mechanisms underlying different progression pathways may ultimately provide diagnostic tools to distinguish which patients will benefit from androgen-deprivation therapy and which may require additional or alternative treatments. Distinguishing downstream AR functions that promote differentiation from those that promote proliferation will lead to development of more targeted treatments. In these endeavors, the Q tract variant mice are a valuable preclinical model for testing therapies directed at the human AR.

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Rapid paper

Length of the human androgen receptor glutamine tract determines androgen sensitivity *in vivo* ☆

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Humanized mouse model

ABSTRACT

A well established functional polymorphism of the human androgen receptor (hAR) is the length of AR's N-terminal glutamine tract (Q-tract). This tract is encoded by a CAG trinucleotide repeat and varies from 8 to 33 codons in the healthy population. Q-tract length is inversely correlated with AR transcriptional activity *in vitro*, but whether endogenous androgen action is affected is not consistently supported by results of clinical and epidemiological studies. To test whether Q-tract length influences androgen sensitivity *in vivo*, we examined effects of controlled androgen exposure in "humanized" mice with hAR knock-in alleles bearing 12, 21 or 48 CAGs. Mature male mice were analyzed before or 2 weeks after orchidectomy, with or without a subdermal dihydrotestosterone (DHT) implant to attain stable levels of this non-aromatizable androgen. The validity of this DHT clamp was demonstrated by similar serum levels of DHT and its two primary 3αDiol and 3βDiol metabolites, regardless of AR Q-tract length. Q-tract length was inversely related to DHT-induced suppression of castrate serum LH ($p = 0.005$), as well as seminal vesicle (SV) weight ($p = 0.005$) and prostate lobe weights ($p < 0.006$). This confirms that the hAR Q-tract polymorphism mediates *in vivo* tissue androgen sensitivity by impacting negative hypothalamic feedback and trophic androgen effects on target organs. In this manner, AR Q-tract length variation may influence numerous aspects of male health, from virilization to fertility, as well as androgen-dependent diseases, such as prostate cancer.

1. Introduction

The androgen receptor (AR) is a ligand-activated transcription factor that is crucial in male sexual differentiation and development (Quigley et al., 1995), including formation of the prostate gland and its late-life pathology (Cunha et al., 2004). A well established genetic polymorphism that influences AR activity is the CAG triplet repeat in *Ar's* exon 1, which encompasses the receptor's N-terminal transactivation domain. This CAG repeat encodes a glutamine tract (Q-tract) that varies in length from 8 to 33 repeats (median 21) in the normal population (Rajender et al., 2007). Pathological Q-tract expansion causes the rare X-linked neurodegenerative disease, spinobulbar muscular atrophy (Kennedy syndrome), resulting from a toxic gain-of-function mechanism, as well as mild androgen insensitivity (Thomas et al., 2006; Lieberman and Robins, 2008).

ARs with shorter Q-tract lengths exhibit increased transactivation of androgen-responsive reporter genes *in vitro* (Chamberlain et al., 1994; Tut et al., 1997; Beilin et al., 2000), supporting the notion that shorter Q-tract ARs are more active at a given ligand level. However, *in vivo* androgen action is complicated by pre-receptor hormone activation, post-receptor co-regulator proteins and systemic and tissue steroid metabolism. *In vivo*, an AR with stronger transcriptional activity (short Q-tract length) would be predicted to produce greater negative feedback suppression of pituitary LH secretion and more potent androgenic trophic effects. However, studies of the relationship between AR Q-tract length and the hypothalamic–pituitary–testicular (HPT) axis in humans have produced conflicting data (Van Pottelbergh et al., 2001; Walsh et al., 2005; Stanworth et al., 2008; Huhtaniemi et al., 2009). Confounding factors that may mask underlying relationships include relatively small effects of Q-tract length, compensation by negative feedback, misclassification of clinical endpoints and interactions with numerous other genetic and physiological pathways.

In order to test directly whether AR Q tract length mediates *in vivo* androgen sensitivity, we used an engineered mouse model

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with human AR (hAR) alleles varying in Q-tract length (Albertelli et al., 2006). In these "humanized" mice, the hAR N-terminal domain (NTD) replaces that of the mouse, eliminating the 15% amino acid difference of the murine NTD as well as its displaced Q tract. Alleles with short (12Q), median (21Q) or long (48Q) glutamine tracts represent the average and extremes in Q-tract length within the human population. These AR alleles in mice produce differences in target gene transcription and prostate cancer progression but systemic effects on the androgen sensitivity have not been investigated in detail (Albertelli et al., 2006, 2008). To determine androgen effects without the confounding influences of variable circulating levels of testosterone (T) or aromatization of T to estradiol (Huhtaniemi et al., 2009), dihydrotestosterone (DHT) implants were used to "clamp" levels of pure androgen following orchidectomy. The short-term use of the DHT clamp paradigm permits causal relationships to be elucidated unambiguously using an open-loop system compared with the inevitable confounding in the native closed-loop system. This reveals the AR Q tract length influence on the key androgen sensitive endpoints of negative hypothalamic–pituitary feedback on gonadotropin secretion and trophic effects on accessory glands.

2. Materials and methods

2.1. Animals and DHT treatment

Mice with "humanized" AR alleles containing polyamino acid tracts of 12, 21 or 48 Qs were generated and genotyped as previously described (Albertelli et al., 2006). All mouse procedures were approved by the University of Michigan Committee on Use and Care of Animals, in accord with the NIH Guidelines for the Care and Use of Experimental Animals. Male mice were orchidectomized at 8 weeks of age and 9 days later treated by subdermal implantation of 0.5 cm silastic tubing filled with ~5 mg crystalline DHT (Singh et al., 1995) for an additional 5 days. DHT was used since it is non-aromatizable unlike testosterone. A DHT dose was chosen, based on previous experience (Simanainen et al., 2009; Allan et al., 2010), to produce partial suppression (~50%) of post-castration increases in serum gonadotrophin levels.

2.2. Sample collection

Mice were killed by cardiac exsanguination under isoflurane anaesthesia. Seminal vesicles were dissected from the urogenital track and freshly weighed both before and after emptying the gland by manual expression of fluid secretions. The amount of secretions was defined as the difference between weights of intact and emptied seminal vesicles. The remaining urogenital track including bladder, urethra, and surrounding prostate lobes was fixed in 4% paraformaldehyde over night at 4 °C for histology. Following fixation, individual prostate lobes were dissected free of fat and connective tissue and weighed separately.

2.3. Hormone assays

All assays were performed in a single batch. Mouse serum LH was analyzed using an immunofluorometric assay as previously described (Jimenez et al., 2005), but using specific antibodies for mLH. The capture antibody used is the anti-LH antibody (5303 SPRN-1, Medix Biochemica, Turku, Finland) and the detection antibody is the anti-LH antibody (MAb 518B7, supplied by Dr. J. Roser, Department of Animal Science, UC Davis (Spearow and Trost, 1987)), directly labeled with a Europium chelate using the DELFIA Eu-labeling kit (Perkin Elmer, City, Country) as per suppliers methodology. For the mLH assay, the detection limit was 0.02 ng/ml, the quantification limit 0.05 ng/ml and the within-assay QC was 6.8% at low (0.25 ng/ml), 4.7% at mid (0.49 ng/ml) and 7.4% at high (1.18 ng/ml) range. Mouse serum FSH was determined using a specific immunofluorometric assay as described and validated previously (Jimenez et al., 2005).

Serum levels of T, DHT and its two principal metabolites 5α-androstane-3α,17βDiol (3αDiol) and 5α-androstane-3β,17βDiol (3βDiol) were measured in extracts of 50 μl (intact) or 100 μl (DHT-treated) of mouse serum by liquid chromatography tandem mass spectrometry (LC–MS/MS) (Harwood and Handelsman, 2009) as adapted for mouse serum and tissues (McNamara et al., 2010). Serum was extracted with 3:2 (volume:volume) of hexane:ethyl acetate fortified with testosterone-1,2,3-d_3 (d_3-T), dihydrotestosterone-16,16,17-d_3 (d_3-DHT), 5α-androstane-3α,17βDiol-16,16,17-d_3 (d_3-3αDiol) and 5α-androstane-3β,17βDiol-16,16,17-d_3 (d_3-3βDiol) as internal standards. The organic layer, separated by freezing the aqueous layer, was dried and reconstituted in 1.2 ml of 20% methanol in PBS prior to injection onto the C8 column for analysis (1 ml). The level of quantification (LOQ) for T, DHT, 3αDiol and 3βDiol were 20, 100, 400 and 400 pg, respectively.

2.4. Histology

Paraffin embedded, fixed prostate lobes were sectioned at 5 μm and slides were stained with hematoxylin and eosin for basic histological analysis.

2.5. Detection of proliferation and apoptosis

Cell proliferation was determined using a proliferating cell nuclear antigen (PCNA) kit (Zymed, San Francisco, CA) and apoptosis by *in situ* detection of Tunel staining of nuclear DNA fragmentation using the ApopTag kit (Chemicon) as previously described (Simanainen et al., 2009). CASTGRID V1.10 (Olympus Corp., Albertslund, Denmark) software was used for unbiased stereological analysis of prostate epithelial proliferation index based on counting of at least 500 cells as previously described (Simanainen et al., 2007, 2009).

2.6. Statistics

Statistical analysis was performed using two-way analysis of variance (ANOVA) with treatment group (intact, castrate, castrate + DHT) and Q-tract length as the main fixed factors using the least significant difference (LSD) method as a post hoc test and suitable linear contrasts for Q-tract trend or specific a priori comparisons. In the case of significant main factor interactions, the simple effects within the main factor were compared by one-way ANOVA with the LSD method as a post hoc test. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL) and NCSS (Kaysville, UT) software. Data is expressed as mean and standard error of the mean (SE) unless otherwise specified and p values less than 0.05 were considered statistically significant.

3. Results

3.1. Validation of the DHT clamp

Mature "humanized" AR males at 8 weeks of age underwent orchidectomy to ablate both the androgenic support of target organs and the negative feedback regulation on gonadotrophins. After 9 days, orchidectomized males were treated with subdermal DHT implants for 5 days to fix the androgen delivery at a constant level. The validity of this DHT clamp was determined by analyzing

the circulating androgen levels using a LC–MS/MS based method tested previously for mouse samples (McNamara et al., 2010). In orchidectomized, DHT-treated males, serum levels of DHT (Fig. 1A) and its metabolites, 3αDiol and 3βDiol (Fig. 1B and C), were similar in mice regardless of AR Q-tract length. Serum T was undetectable in all samples from orchidectomized males, with or without DHT treatment. In the intact males, median serum T did not significantly differ with Q-tract length, with the median (range) being 0.11 (0.03–13), 0.13 (0.01–0.98) and 0.38 (0.13–1.5) ng/ml for 12Q, 21Q and 48Q, respectively (Fig. 1D).

3.2. AR-dependent negative feedback regulation of serum LH, but not FSH, is influenced by AR Q-tract length

DHT-induced suppression of castrate LH levels was greatest for the shorter Q-tract length ($p < 0.005$ test for trend), with DHT-suppressed serum LH levels being 6%, 26% and 48% of castrate levels in 12Q, 21Q and 48Q males, respectively (Fig. 2A). Furthermore, following DHT treatment, the suppressed LH levels remained markedly higher than intact levels in 48Q males (65% higher than intact) while 21Q males were restored to intact levels (15% lower). In 12Q males the DHT treatment reduced serum LH levels below the intact level (70% lower). AR Q-tract length had no influence on serum LH or FSH in intact or untreated orchidectomized mice or on serum FSH levels in DHT-treated orchidectomized mice (Fig. 2B).

3.3. Androgen-dependent sex accessory organ weights correlate with AR Q-tract length

Seminal vesicle (SV) weights in intact males followed the Q-tract length order 12Q > 21Q > 48Q. The trend was highly significant for full (tissue + secretion; $p < 0.001$) and empty (manually emptied gland; $p = 0.008$) SV weights as well as SV secretions ($p = 0.001$). Therefore the smaller full SV weights in 21Q (82 ± 7% of 12Q) and 48Q (60 ± 6% of 12Q) males were due to AR Q-tract effects on both tissue weight and secretion volume (Fig. 3A–C). In all males, orchidectomy-induced involution reduced the full SV mass by an average 90% of the intact weight and followed the Q-tract order (12Q > 21Q > 48Q). The tissue weight was reduced to 80% of intact weights and significantly ($p = 0.001$) followed the trend in Q-tract length. The volume of SV secretion was mostly non-detectable following castration. DHT treatment after castration restored SV weights to about 60% of the respective intact weights within the 5 days of treatment. Moreover, the SV weights followed the Q-tract order with a significant trend for full ($p < 0.001$) and empty ($p = 0.008$) glands and a non-significant trend for weight of the secretions ($p = 0.063$).

The prostate anterior (AP), ventral (VP) and dorsolateral (DLP) lobes were analyzed separately from intact, castrate or castrate + DHT treated hAR male mice. In intact males, there was no significant Q-tract dependent trend for prostate lobe weights, although the VP and AP lobes were smallest in 48Q males, suggestive of their lower androgen responsiveness (Fig. 3D and E). As expected, orchidectomy reduced (<35% of intact), and short-term DHT administration restored (>60% of intact), the weight of all prostate lobes. For both castrate and DHT-treated orchidectomized mice, AR Q-tract length influenced prostate lobe regression and regrowth consistently and significantly (12Q > 21Q > 48Q; $p < 0.05$). The trend was most prominent following DHT stimulation for all lobes ($p \leqslant 0.005$) with the greatest regrowth achieved for the DLP. Weights in 12Q, 21Q and 48Q mice were 119%, 91% and 82% relative to the intact lobe, while the VP weights were 91%, 82% and 70%, and AP weights 95%, 68% and 63%, relative to intact weight. Similarly, following castration the weights correlated inversely with Q-tract length (12Q > 21Q > 48Q), with significant

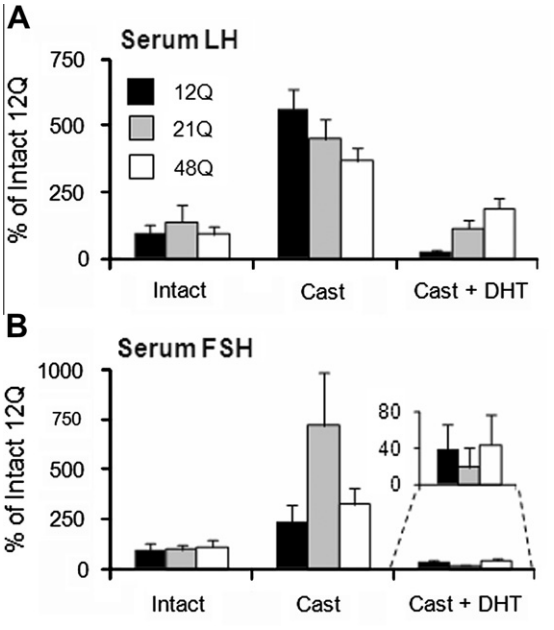

Fig. 2. Serum gonadotrophin levels: feedback on gonadotrophin secretion in Q tract variant mice. Serum gonadotrophins LH (A) and FSH (B) were analyzed using mouse-specific immunofluorometric assays. The levels are shown, relative to intact 12Q males, for intact, castrated (9 days) + DHT (5 days) or castrated (14 days) mice. The LH and FSH levels were similar between intact males with different AR Q-tract lengths. The DHT treatment restored the serum LH from castrate level to intact in 12Q and 21Q males, but not in 48Q males, with a significant ($p = 0.005$) trend observed between Q-tract length and serum LH. Castration significantly increased the LH levels in all males, with the magnitude of increase following the Q-tract length with 12Q highest and 48Q lowest. Serum FSH levels were not statistically different between 12Q, 21Q and 48Q males in any treatment group. Data shown as mean ± SE. $N = 9–10$ for all groups. For reference, the intact levels are shown as Supplemental data in Table 1.

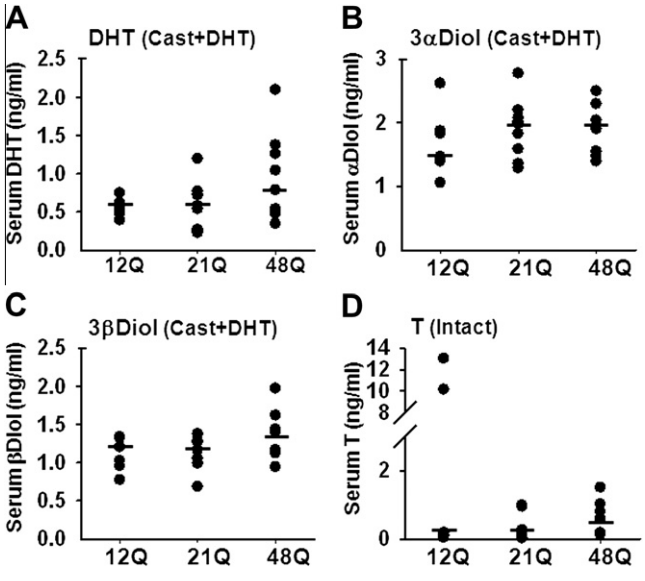

Fig. 1. Serum steroid hormone levels: the Q-tract length does not significantly influence serum hormone levels. Serum from hAR 12Q, 21Q and 48Q mice was analyzed for DHT (A), 3αDiol (B) and 3βDiol (C) levels in castrated and DHT-treated males and for T in intact males (D), by the LC–MS/MS based method. DHT, 3αDiol and 3βDiol were undetectable in intact males, and no T was detected in serum of DHT-treated males (not shown). Each dot represents the serum steroid concentration of one animal. The median concentrations are shown by horizontal lines. $N = 8–10$ for all groups.

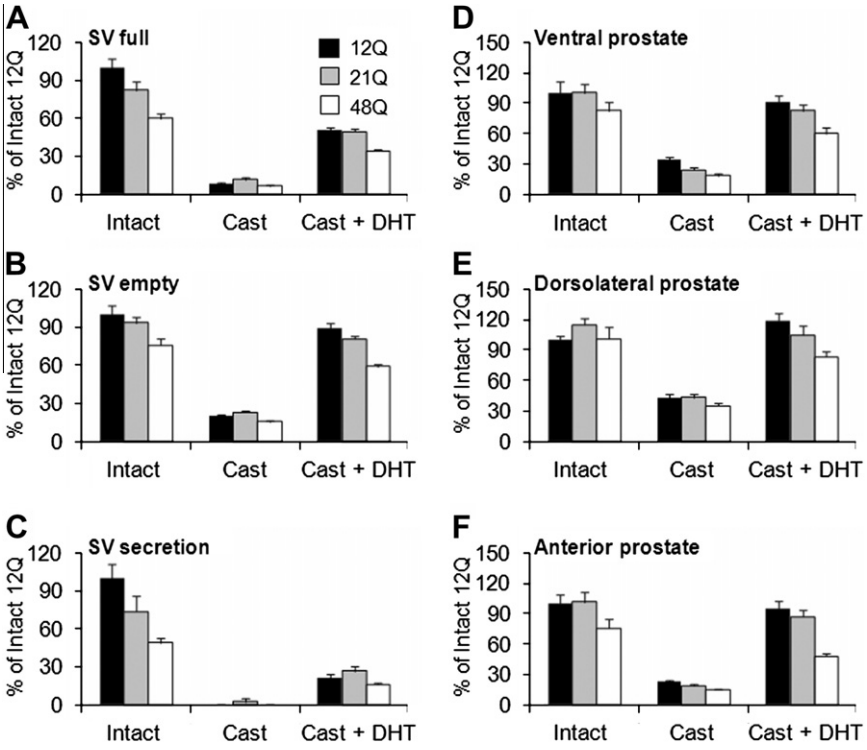

Fig. 3. Seminal vesicle and prostate lobe weights: Q tract length affects AR trophic function. Full seminal vesicle (SV; A), emptied SV (manually emptied; B) and secretion (full SV – tissue weight; C) weights shown as relative (%) to intact 12Q weights. Relative data is shown for intact, castration (9 days) + DHT (5 days) treatment or castration (14 days) only. The SV weights followed the expected sensitivity difference with 48Q males having smaller androgen dependent sex accessory organs followed by 21Q and 12Q males. Data shown as mean ± SE. $N = 9–10$ for all groups. For reference, the intact levels are shown as Supplemental data in Table 1. Ventral (VP; D), dorsolateral (DLP; E) and anterior (AP; F) prostate lobe weights in hAR mice with 12Q, 21Q or 48Q-tract lengths, shown relative (%) to intact 12Q weights. Relative data is shown for intact, castration (9 days) + DHT (5 days) treatment or castration (14 days) only. The prostate weights followed the expected sensitivity difference with 48Q males having smaller androgen dependent sex accessory organs followed by 21Q and 12Q males.

trends observed for VP ($p = 0.004$) and AP ($p = 0.013$), and a non-significant ($p = 0.075$) trend in DLP (Fig. 3D and E).

3.4. Proliferation and apoptosis reveal lobe-specific AR Q-tract dependence in DHT responses

Epithelial proliferation was quantified by stereological determination of the proportion of PCNA immunopositive cells within the prostate epithelia in intact and DHT-treated males (Fig. 4A–C). In intact mice the prostate epithelial proliferation was low (<3% of epithelial cells) in all prostate lobes for all groups and was not detected after castration (not shown). With 5 days of DHT treatment, the epithelial proliferation increased significantly ($p < 0.05$) above intact level in all lobes. Particularly in the VP, epithelial proliferation was dependent on the Q-tract length and was higher in 48Q and 21Q males compared to 12Q males. In DLP and AP the epithelial proliferation did not differ significantly between the Q-tract lengths (Fig. 4A).

Prostate epithelial apoptosis was determined by stereological quantification of Tunel-positive epithelial cells. After castration, epithelial apoptosis was markedly increased above levels in intact mice and depended on Q-tract length, with 48Q significantly ($p = 0.002$) higher than 12Q and 21Q (Fig. 4D–F). Apoptosis was very low (<1%) or undetectable in prostate lobes of all intact and DHT treated males (not shown).

4. Discussion

By use of a DHT clamp, we show that systemic AR activity correlates inversely with Q-tract length and can significantly influence androgen dependent physiological processes. In the humanized AR mice, acute DHT-induced suppression of post-castration serum LH level is greatest in 12Q males and least in 48Q mice. Similarly, the post-castration re-growth of the prostate and seminal vesicles positively correlates with the AR Q-tract length. The experimental paradigm of the DHT clamp eliminates differential hormone metabolism as an explanation for Q tract effects and allows detection of small differences that may be less evident at the molecular level in intact mice (Albertelli et al., 2006).

These findings agree with some epidemiological studies showing AR Q-tract length dependent variation in serum LH and/or testosterone levels (Walsh et al., 2005; Stanworth et al., 2008; Huhtaniemi et al., 2009; Lindstrom et al., 2010). However, although androgen sensitivity *in vivo* is largely a product of androgen levels, AR expression, and transcriptional activity, it can be significantly modified by other hormones and physiological as well as study limitations that are difficult to assess or control in clinical settings. Thus, there are conflicting results on whether AR Q-tract length affects clinical endpoints (Van Pottelbergh et al., 2001; Zitzmann et al., 2001). Complexity is evident in the present study since a Q-tract length effect is not seen for serum FSH, which is subject to more powerful regulation by inhibin (Hayes et al., 2001a) and estradiol (Hayes et al., 2001b), or for serum LH in intact males (with a closed loop feedback system) or orchidectomized mice (with complete androgen deficiency). However the effects that are seen here are androgen and AR mediated. Interestingly, the AR Q-tract affect appears to influence post-castration LH levels. It may be that the LH promoter is subject to differing intensity of androgenic negative feedback dictated by the Q tract length. Some of the differences from clinical results may be species-specific. For example, serum T in mice varies greatly between individuals and

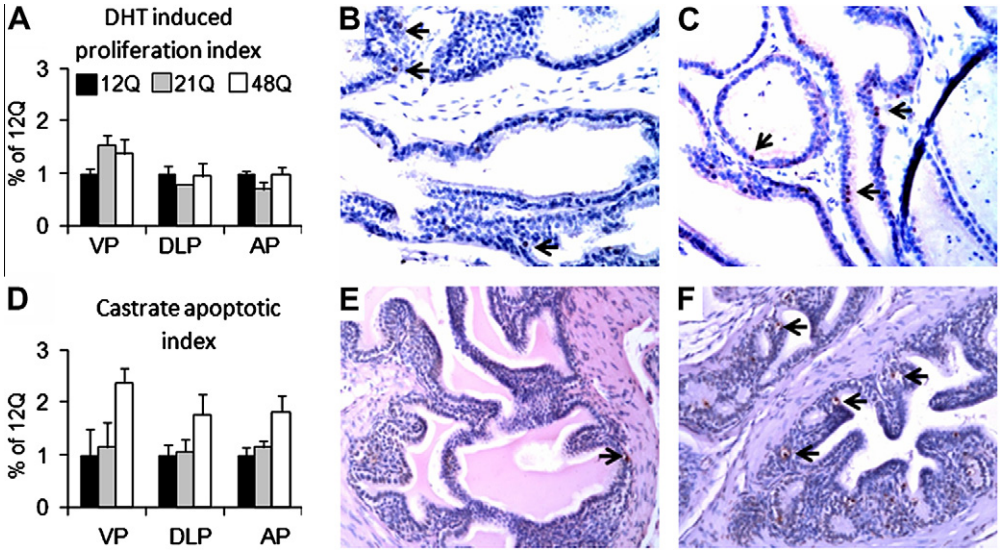

Fig. 4. Prostate epithelial cell proliferation and apoptosis: proliferating cell nuclear antigen (PCNA) immunostaining was performed to detect proliferative cells (epithelial proliferation index) in VP, DLP and AP and quantified by stereology. (A) The DHT-induced proliferation in 12Q, 21Q and 48Q males is shown relative to 12Q. Representative photos of PCNA immunopositivity in VP of 12Q (B) and 48Q (C) males. Examples of positive staining are indicated by arrows. Tunel *in situ* staining was performed to detect apoptotic cells (apoptotic index) following castration in VP, DLP and AP, and quantified by stereology. (D) The apoptotic index in castrated 12Q, 21Q and 48Q males is shown as relative to 12Q. Representative photos of Tunel positivity in AP of 12Q (E) and 48Q (F) males. Examples of positive staining are indicated by arrows.

over time, in part due to lack of the dampening effects of circulating SHBG as in men (Bartke and Dalterio, 1975; Coquelin and Desjardins, 1982). Even with more stable serum T in man, large sample sizes are required to demonstrate differences in negative feedback set-point (Krithivas et al., 1999; Crabbe et al., 2007; Huhtaniemi et al., 2009).

Complementary evidence for Q-tract length effects on androgen sensitivity is provided by the effects of DHT on seminal vesicle and prostate weights after orchidectomy. The weights significantly follow the Q-tract length order, with greatest growth in 12Q and least in 48Q mice. These findings are similar to the influence of CAG repeat length on prostate growth in hypogonadal men treated with T (Zitzmann et al., 2003). Prostate epithelial cell renewal is androgen dependent (Cunha et al., 1987; Heinlein and Chang, 2004), but the level of proliferation in prostate lobes 5 days after androgen replacement does not follow the expected Q-tract length order. However, while androgen action in the prostate is often thought to be only proliferative and anti-apoptotic, recent studies reveal an anti-proliferative role of androgens in promoting epithelial cell differentiation and thereby suppressing proliferation (Simanainen et al., 2007; Wu et al., 2007). Transient rapid induction of proliferation upon androgen replacement followed by a steady low level of epithelial proliferation has been shown previously in rodent prostate (Chen et al., 1996). Therefore, the 12Q AR may be more potent in promoting differentiation following androgen replacement leading to lower epithelial proliferation compared to 21Q and 48Q. However, the effects on tissue weights demonstrate that although the Q-tract effects may be small, sustained subtle differences in AR activity may be cumulative over time. These slight differences in AR activity over a lifetime may impact androgen dependent organs and susceptibility to late-life hormone-dependent diseases.

This study clarifies some previous conflicts in epidemiological studies and suggests that the effects of Q-tract length are genuine but of a magnitude that may be masked by diverse genetic and/or environmental factors. This is especially problematic for observational studies that typically are not randomized or adjusted to control for unknown as well as known risk factors. In the homogeneous genetic background of the hAR mice, Q tract length effects on prostate cancer progression and response to androgen deprivation therapy are more readily detected than in human populations (Albertelli et al., 2008). Prostate tumors arise earlier in 12Q than in 48Q mice, but disease progresses slowly, with a well-differentiated phenotype, especially following castration. Along with data here, this suggests that AR Q-tract length affects may be most significant in situations of low or changing androgen levels, as in prostate cancer treatment or in aging men in general (Kaufman and Vermeulen, 2005).

In conclusion, the humanized AR mouse model offers direct experimental evidence that androgen sensitivity is reduced with increasing Q-tract length, indicated by reduced negative feedback on LH secretion and reduced trophic effects on sex accessory glands. Future epidemiological studies may resolve Q-tract length effects if the groups studied are sufficiently well defined, homogeneous and large. Furthermore, while modest differences in AR Q tract length may not measurably affect most aspects of male health, there may be greater affects in individuals with lengths at the extremes of the normal range or when circulating hormone levels are changing, such as during puberty, aging or treatment with exogenous androgens.

Disclosure summary

Authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2011.05.011.

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Review

Androgen receptor gene polymorphisms and alterations in prostate cancer: Of humanized mice and men

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ABSTRACT

Germline polymorphisms and somatic mutations of the androgen receptor (AR) have been intensely investigated in prostate cancer but even with genomic approaches their impact remains controversial. To assess the functional significance of AR genetic variation, we converted the mouse gene to the human sequence by germline recombination and engineered alleles to query the role of a polymorphic glutamine (Q) tract implicated in cancer risk. In a prostate cancer model, AR Q tract length influences progression and castration response. Mutation profiling in mice provides direct evidence that somatic AR variants are selected by therapy, a finding validated in human metastases from distinct treatment groups. Mutant ARs exploit multiple mechanisms to resist hormone ablation, including alterations in ligand specificity, target gene selectivity, chaperone interaction and nuclear localization. Regardless of their frequency, these variants permute normal function to reveal novel means to target wild type AR and its key interacting partners.

Contents

1. Introduction

The androgen receptor (AR) is a crucial regulator of male physiology and orchestrates the development and function of the prostate gland. Cancer of the prostate is a major health problem, confounded in etiology by numerous genetic and environmental factors (Nelson et al., 2003). Despite the marked complexity and heterogeneity in the origin and course of prostate cancer, early stages of tumor growth depend on androgen and, despite therapies aimed at depleting hormone and blocking receptor transactivation, recurrent disease depends on reactivation of AR (Scher and Sawyers, 2005). Just as cancer cells evolve over time due to both intrinsic and extrinsic forces, so AR function transitions from maintaining homeostasis to promoting oncogenesis (Litvinov et al., 2003). Given AR's key role in these processes, variation in the structure or expression of the *Ar* gene is logically suspected of influencing initiation or progression of disease. Extensive

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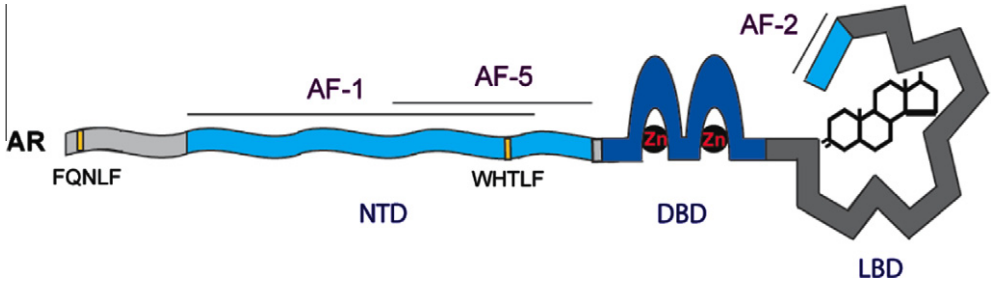

Fig. 1. Structural and functional domains of the AR. Diagrammed are the largely unstructured amino terminus (NTD), zinc-chelating fingers of the DNA binding domain (DBD), and the ligand binding domain (LBD) that is comprised of 12 alpha helices, with dihydrotestosterone (DHT) depicted in the pocket. Relative positions are indicated for motifs involved in N–C interaction (FxxLF, WxxLF), and activation functions that depend on ligand binding (AF-2) or are ligand-independent (AF-1, AF-5). The polymorphic Q tract begins at amino acid 58 in hAR, which is about 920 amino acids in length.

investigation of *Ar* germline polymorphism and somatic mutation has produced inconsistent results on the importance of AR genetic variation in prostate cancer. Despite the controversies, many insights into wild type receptor function have been derived from analysis of genetic variants implicated in disease.

In order to use experimental tools to test the role of AR variants in prostate cancer, and to avoid the confounding heterogeneity of man, we established a mouse model in which the murine *Ar* gene was converted to the human sequence by germline recombination (Albertelli et al., 2006). The DNA and ligand binding domains (DBD, LBD) of human and mouse ARs are identical, with a few amino acid differences in the hinge region (Fig. 1). However the large amino termini (NTD) encompassing the major transactivation domains differ by 15% in protein sequence as well as in position and extent of polymorphic polyglutamine (Q) and polyglycine (G) tracts. Most of the NTD is encompassed within *Ar's* large first exon, allowing mouse and human sequences to be swapped by knock-in technology resulting in a locus in which hAR is expressed under murine regulatory control. This "humanized" model allows us to assess effects of germline polymorphisms on disease phenotypes (Albertelli et al., 2008) and to evaluate relationships between somatic mutation and treatment regimen (O'Mahony et al., 2008). Comparison to patient data directly substantiates the relevance (Steinkamp et al., 2009).

Here we present findings pertaining to controversies in the role of the AR polyQ tract and *Ar* gene mutations in prostate cancer. We summarize recent key studies in the literature but focus primarily on results from our laboratory, including data from both mouse and human prostate cancer patients. More comprehensive reviews and discussion of implicated mechanisms have recently been published (Buchanan et al., 2009; Robins, 2009).

2. AR polymorphisms and prostate cancer

AR's NTD coordinates interactions with coregulators and nonreceptor transcription factors that are critical for both ligand-dependent and -independent transactivation (Robins, 2004; Shen and Coetzee, 2005). The N-terminal polyQ tract, encoded by a CAG repeat, was first linked to pathology in Kennedy disease. Abnormal expansion of this tract underlies late-onset atrophy of spinal and bulbar motor neurons due to misfolding and aggregation of the mutant AR, compounded by partial loss of function (Lieberman and Robins, 2008). Transfection assays ascribe the latter to an inverse correlation between Q tract length and AR transcriptional strength (Mhatre et al., 1993). Sensitivity to Q tract length difference within a "normal" range (9–37 CAGs) is likely due to effects on overall AR structure rather than to site-specific interactions (Irvine et al., 2000). Shorter Q tracts enhance AR's critical intramolecular N-C interaction, allowing response to lower androgen concentrations and more effective coactivation by p160 proteins (Buchanan et al., 2004; Wang et al., 2004). Because Q tract length affects AR activity in transfection, associations between such variation and male traits have been sought. Substantial evidence links AR Q tract length to male fertility (Davis-Dao et al., 2007) and in hypogonadal men to response to testosterone replacement (Zitzmann et al., 2004). Association of Q tract length with prostate cancer, where a more active AR might promote oncogenesis, remains controversial.

2.1. Human genetic and genomic studies of the CAG repeat and prostate cancer

That short Q tract ARs increase prostate cancer risk was first conjectured based on their greater prevalence in the high-risk African-American population (Coetzee and Ross, 1994), and gained credence from their increased transcriptional activity *in vitro* and evidence of Q tract contraction in malignant but not benign prostate cells (Schoenberg et al., 1994; Alvarado et al., 2005). Epidemiological studies initially found association between fewer CAG repeats and increased risk or more aggressive disease (e.g., (Irvine et al., 1995; Giovannucci et al., 1997; Stanford et al., 1997)). However results vary with ethnic group and most recent studies find no association with risk (e.g., (Lindstrom et al., 2010; Price et al., 2010)). Conflicting conclusions stem from many aspects of study design, including small sample sizes, differences in environment, genetic admixture, ascertainment bias (including diagnosis before or after the age of PSA testing) and differences in tract length cut-off points. Regardless of these variables, modern genotyping technology applied to large case-controlled groups fails to substantiate *Ar* CAG tract length as a predictor of prostate cancer (Price et al., 2010). *Ar* may be more comparable to a quantitative trait, the effect of which depends on variation at other loci and on environmental interactions. In support of this, allelic differences in genes participating with *Ar* in the androgen axis, including those encoding enzymes of testosterone synthesis (cytochrome P450c17) and conversion (steroid-5-α-reductase type 2), reveal haplotypes that do differ significantly in prostate cancer risk (Lindstrom et al., 2006).

While *Ar*'s CAG repeat may not impact risk of prostate cancer, it may affect other aspects of disease that are subject to AR function, such as progression or treatment response. Several studies support a relationship between Q tract length and serum levels of testosterone and estradiol, likely due to the effect of differential AR strength in systemic feedback control (Huhtaniemi et al., 2009; Lindstrom et al., 2010). Whether Q tract variation might also impact intratumoral hormone levels, or their effect, is of interest in light of increasing evidence of prostatic androgen synthesis (Montgomery et al., 2008). Furthermore, CAG repeats are inherently unstable and Q tract contraction is found in malignant prostate cells but not in adjacent normal tissue (Alvarado et al., 2005). Heterogeneous

somatic variation in Q tract length may play a role in the increased AR activity that occurs in prostate cancer recurrence. Given the complex and contradictory data on the role of the Q tract in prostate cancer, we turned to a mouse model to gain biological insight.

2.2. Effects of polyQ tract length on AR biology: insights from genetically engineered mice

To investigate the effect of AR Q tract length on prostate cancer, we developed knock-in strains with *hAr* alleles containing 12, 21 or 48 CAG repeats (Albertelli et al., 2006). All mice in this allelic series (referred to as AR12Q, AR21Q, or AR48Q mice) are grossly normal and have similar fertility, with no neurological problems evident in the AR48Q mice (much longer CAG repeats are needed to model neurodegeneration) (Lieberman and Robins, 2008). Transactivational differences due to Q tract length are however detectable in expression of prostatic AR target genes. Moreover, allele-dependent responses following hormonal perturbation corroborate a role of AR Q tract length in systemic androgen sensitivity (*ms. in press*). Therefore these mice model human AR Q tract length variation within a "normal" range.

Introducing a prostate-targeted SV40 T-antigen (Tag) oncogene by crossing to TRAMP mice reveals differential effects of the humanized *Ar* alleles on cancer initiation, progression and response to androgen ablation (Albertelli et al., 2008; Robins et al., 2008) (Fig. 2). Mice with short Q tract ARs develop prostatic intraepithelial neoplasia (PIN) earlier than mice with median (21Q) or long (48Q) tracts. However, the resulting AR12Q tumors are well differentiated, progress slowly and allow longer survival of AR12Q than AR21Q mice. Remarkably, following androgen ablation Q tract length effects are in directions opposite to those in hormone intact mice. Castrated AR12Q mice develop tumors later than the other strains and live longer than their intact littermates, indicating good response to treatment. In contrast, AR48Q-TRAMP mice fail to benefit from androgen ablation. Detection of these divergent responses due to a modest variation in AR activity is more evident on a homogeneous genetic background. This suggests that *Ar* variation at the extremes of the normal range may also impact certain cases of human disease.

Effects in TRAMP mice are complicated by the AR dependence of the oncogenic transgene, but that is unlikely to account for all differences noted. In some regards this complexity mimics the androgen-responsiveness of *TMPRSS2-ERG* fusion genes that are critical in progression (Tomlins et al., 2007). Some of the tumor characteristics may reflect varying AR functions at different disease stages

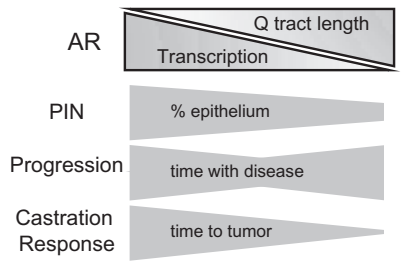

Fig. 2. Mouse prostate cancer parameters vary with AR Q tract length. At top is modeled the inverse correlation between Q tract length and AR transcriptional activity based on transfection assays. In 12 week old TRAMP mice, the percent of prostate dorsal lobe epithelium involved in PIN is greatest in 12Q and least in 48Q mice. Despite this apparently earlier initiation, tumors in 12Q mice progress slowly (leading to a longer time with disease) and have a well-differentiated phenotype; tumors in 48Q mice progress slowly after a long lag to initiation. Following androgen ablation, 12Q mice develop palpable tumors much later than intact littermates, whereas there is no difference in time to palpation for 48Q mice. This qualitative depiction summarizes data in Albertelli et al. (2008).

and in distinct cell types (e.g., stroma vs. epithelia). For example, it is reasonable to predict that weak AR action in TRAMP would enhance castration response due to loss of the androgen-driven Tag oncogene. Instead, AR48Q castrates fare worse after castration, perhaps because weak stromal AR fails to repress growth of preexisting androgen-independent cells. Similarly, the hypersensitive AR12Q may more ably maintain epithelial differentiation despite hormone ablation and thereby slow tumor growth. Results from these mice support further investigation of an influence of AR Q tract variation in later stage human disease. In addition these mice provide a unique experimental model in which to define downstream events that predict response to therapy. Our future studies will elucidate mechanisms by which Q tract length impacts ligand-independent AR function and define corresponding pathophysiology in models that more accurately reflect human disease.

3. AR alterations in prostate cancer

Somatic mutation is a hallmark of cancer, whereby uncontrolled growth stems from loss of tumor suppressor genes and aberrant function of oncogenes. Coincident genomic instability leads to a mutator phenotype, in which each cancer cell may come to harbor hundreds of mutations (Bielas et al., 2006). Most mutations likely have little phenotypic effect and are considered "passengers", but some may be "drivers" that provide a growth advantage or promote metastasis (Greenman et al., 2007; Jones et al., 2008). Recent studies using genomic tools provide quantitative data reinforcing this view. A high-throughput interrogation of 1000 tumors for known mutations indicates that very few mutations occur frequently but mutations in several oncogenes co-occur (e.g., *PIK3CA* mutations coincide with mutations in *KRAS*), highlighting sensitive pathways rather than individual genes as potential therapeutic targets (Thomas et al., 2007). Genome-wide characterization shows that mutation rates and genes mutated vary substantially across tumor types and subtypes (Kan et al., 2010). Interestingly, mutation rates appear relatively low in prostate cancer, where ~75% of tumors carry *TMPRSS2-ERG* gene fusions (Tomlins et al., 2007). In contrast, in a lung tumor next-generation sequencing revealed a wide variety of somatic mutations, with differing patterns in expressed vs. nonexpressed genes (Lee et al., 2010). While most of these mutations are likely passengers some, such as those occurring in kinase genes, appear to be drivers because they evidence distinct selective pressures within the tumor environment. For prostate cancer, numerous mutations have been identified in *Ar* and many are gain of function (Buchanan et al., 2009). However, most aberrant ARs have been identified in small studies and the effects of these mutations have largely been determined *in vitro* (a notable exception is AR-E231G – see below). Therefore *Ar* mutation frequency and the functional significance of mutant ARs in disease remain unclear. As with the Q tract controversy, genomic approaches and mouse models are producing new insights.

3.1. AR mutations – driving in heavy traffic

Somatic gain-of-function mutations in AR are compelling because their actions implicate mechanisms of disease recurrence in castration resistant prostate cancer (CRPC). Mutations have been identified in tumors that alter ligand specificity, increase sensitivity to low androgen levels or allow ligand-independent activation, all proposed as mechanisms of therapy resistance (Feldman and Feldman, 2001). The paradigm of a mutant that could evade treatment is AR-T877A, first identified in the LNCaP cell line and subsequently in several advanced prostate cancers (Taplin et al., 1995). The subtle shift in LBD structure due to T877A permits various noncanonical ligands, including the antiandrogen

hydroxyflutamide, to confer an active conformation (Hur et al., 2004). Other LBD mutations that result in promiscuous ARs, such as H875Y, also harbor clear potential for hormone resistance. Mutations in the NTD could lead to androgen-independent AR activation but fewer examples have been identified since sequencing this region is impeded by its high GC content and polymorphic repeats. Moreover, most studies have examined small numbers of patients using methods that do not detect low frequency *Ar* mutations, thus underestimating the heterogeneity of prostate cancer between individuals as well as within the tumor. Nevertheless, *Ar* mutations have been reported in up to one-third of tumor samples and most commonly following treatment, suggesting they may be selected by therapy (Taplin et al., 1999). The use of microdissection and laser capture has greatly improved the ability to detect *Ar* mutations (Marcelli et al., 2000; Alvarado et al., 2005).

Recent comprehensive surveys with large sample sizes support the common occurrence of somatic AR alterations in prostate cancer progression, with the most frequent event being gene amplification (Holcomb et al., 2009; Liu et al., 2009; Taylor et al., 2010; Robbins et al., 2011). Targeted exon sequencing of genes of high interest to prostate cancer identified promiscuous AR mutants, including AR-T877A and AR-H875Y, in 5–10% of the samples (Taylor et al., 2010; Robbins et al., 2011). The methodology however would not have detected variants present in a subpopulation of cells or prematurely truncated ARs due to nonsense mutation or alternative splicing. The latter has become a focus of recent attention (Jagla et al., 2007; Dehm et al., 2008; Hu et al., 2009). There is surprising diversity in these splice variants, many of which lack C-terminal sequences and thereby could encode constitutive ARs that drive castration resistance. However, most variants in tumor cells are low in abundance relative to correctly processed *Ar*, appear in response to hormone withdrawal and some may require wild type receptor to function, making it unclear whether they play a dominant role in resistance to therapy (Watson et al., 2010). Recently in a cell-based model, truncated ARs due to intragenic rearrangements have been shown to promote prostate cancer progression (Li et al., 2011). As with mutant ARs, further study of alternatively spliced forms may shed critical light on how wild type receptor drives progression despite antiandrogen therapy.

3.2. AR mutation profiling in prostate tumors of mice and men

In mice, genetic background, environment and treatment can be controlled, allowing better assessment of the prevalence of *Ar* mutations in prostate cancer and the extent to which variant ARs reflect tumor biology. Prior study of intact (untreated) vs. castrated (androgen ablated) TRAMP mice found that missense *Ar* mutations in primary tumors vary with hormonal status (Han et al., 2001). To obtain more direct evidence that this is due to the selection pressure of therapy, we used the humanized AR mice to ask whether distinct types of mutations arise with different treatments. hAR21Q-TRAMP mice were either untreated, castrated at 12 weeks (when PIN but not overt cancer is present), or treated with different antiandrogens upon detection of a palpable tumor. Comparison of bicalutamide to flutamide is particularly informative – the bulkier bicalutamide more significantly distorts the AR LBD and so unlike flutamide is less likely to impose an agonist conformation from a single amino acid change (e.g., AR-T877A). To identify potentially functional AR mutants that might be present in only some cells, the equivalent of 20 *Ar* mRNAs per tumor were sequenced from reverse-transcribed, PCR-amplified and subcloned cDNAs (O'Mahony et al., 2008). *Ar* sequences from testes of these mice provide an estimate of technical error rate; sequence differences in tumor samples were twice as frequent as in testes. To highlight changes more likely to be actual mutations, only those that recurred within a tumor or occurred in multiple tumors were studied. Since only one codon change recurred in testis this conservative view minimizes methodological errors. By this approach, missense mutations in *Ar* that repeat within or between tumors occur at a rate of ~0.5/10 Kbp, with most having an overall tumor frequency of less than 10% of the cells.

Recurring missense AR mutations are least frequent in tumors from untreated mice, evidencing effects of treatment and corroborating clinical observations (Taplin et al., 1999). Mutations following castration occur mostly in the NTD, whereas flutamide-treated mice have more mutations in the C-terminal portion, including some premature stop codons that could lead to constitutively active ARs. Moreover, flutamide-treated mice have the most treatment-specific mutations. Bicalutamide treatment, as expected, led to fewer mutations that primarily occurred in the NTD. Many of the AR mutations in mouse prostate tumors cluster to regions found previously mutated in patients and are generally distinct from loss-of-function mutations identified in Androgen Insensitivity Syndrome (AIS) (Buchanan et al., 2009). Many mutations cluster near the most highly conserved segment of the LBD that affects ligand specificity (Fenton et al., 1997). Some of these that are known to be loss of function as germline mutations are surprisingly active in transfection assays. This suggests that mutations that are null in development may display partial or altered function in other contexts, such as the tumor environment (see below).

To compare directly in man whether mutations differ in number or type after treatment, and whether distinct antagonists select for different mutations, we performed a similar analysis with a set of high quality samples of metastases from either hormone-naïve patients or those treated with only one antagonist (flutamide or bicalutamide) (Steinkamp et al., 2009). As with the mouse samples, the equivalent of 20 *Ar* mRNAs were sequenced and similar base change rates were found. Interestingly, despite having fewer

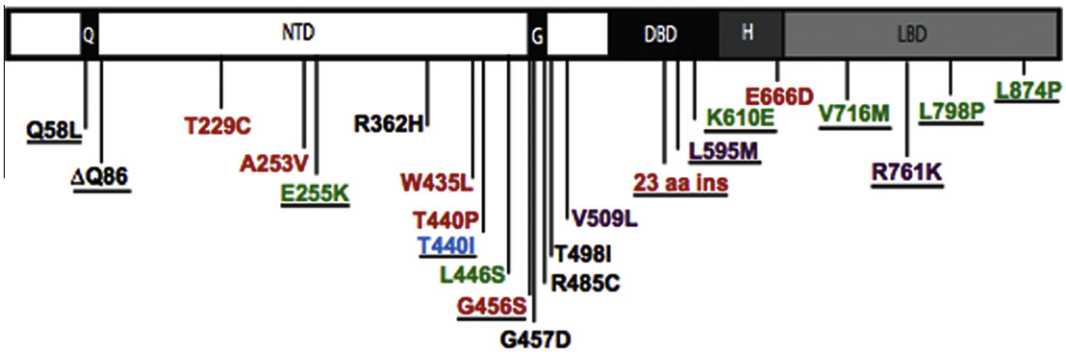

Fig. 3. Recurring AR mutations from human prostate cancer metastases. Missense mutations found in more than one tumor or multiple times in a single tumor are shown; those occurring multiple times in a single patient are underlined. Color code for treatment groups is as follows: green – flutamide, purple – bicalutamide, orange – both antiandrogens, blue – no endocrine treatment, black – both treated and untreated.

human than mouse tumors, more mutations recurred at higher rates (both in more tumors and at higher levels per tumor), providing stronger evidence of treatment selection. In general, mutations occurring at low frequency but in multiple tumors are located in the NTD. In contrast, mutations present in several clones from a single patient tend to be case-specific, not restricted by AR domain and reflective of treatment differences (Fig. 3). Few mutations recur in hormone-naïve samples. The "mutator phenotype" is not equal between samples, with less than half of the tumors accounting for most of the mutations.

Several similarities emerge from our mouse and human *Ar* mutation profiling, including comparable overall mutation frequencies and fewer mutations in hormone-naïve tumors. The variety of recurring mutations suggests that selective value may stem from numerous rare mutations rather than a few common ones, with detection of rare events dependent on sequencing methodology. A significant difference between the mouse and human studies is that more mutations were present at higher frequencies in man. This may reflect the longer time with disease in man allowing a greater period for selection to operate (Steinkamp et al., 2009). Furthermore, human metastases are more clonal in nature than heterogeneous mouse primary tumors. In fact, recent analyses using next generation sequencing suggest somatic genetic variation and selection play a greater role in cancer ontogeny than previously suspected (Gottlieb et al., 2010). Even silent base changes are not random, suggesting selection for greater AR activity may operate at the level of efficiency of translation or co-translational protein folding. Below specific examples illustrate how *Ar* mutations may be functionally relevant to prostate cancer progression.

3.3. Mutant ARs exploit multiple mechanisms to evade therapy

Mutations found in numerous studies cumulatively highlight AR domains within which different mutations may affect a common function and produce a similar phenotype (Buchanan et al., 2009). Further evidence that these mutant ARs may not be simply bystanders is inherent in the means they may use to evade therapy and is reinforced by finding fewer additional mutations in tumors with prevalent variant ARs. For example, the human tumor in which we identified the promiscuous AR-V716M (below) had only one other missense mutation (Steinkamp et al., 2009). Similarly, few AR mutations recur in xenograft tumors originating from VCaP cells that have amplified *Ar* – additional variation may have little value when high levels of wild type AR are attained (*O'Mahony and Robins, unpublished*).

3.3.1. Promiscuity is priceless: AR-V716M

A mutation most clearly supporting treatment selection is V716M, an alteration known to create a permissive receptor responsive to a wide array of hormones and antagonists (Culig et al., 1993). This mutation was present in all 20 *Ar* clones sequenced from the lung metastasis of a flutamide-treated patient whose normal tissue DNA was wild type (Steinkamp et al., 2009). Two additional metastases from this patient also yielded only the mutant sequence, indicating a clonal population carrying AR-V716M accounted for these three metastases and therefore arose either early in metastatic invasion or within the primary tumor itself. The absence of other *Ar* mutations recurring in this patient suggests this variant was effective enough to reduce selective value of other mutations. While fixation of a mutation like V716M may be relatively rare, many cancers may have subsets of cells with different mutations that together provide a similar growth advantage. Interestingly, the patient with AR-V716M survived 19 years beyond diagnosis. It is tempting to speculate that in this case residual activity of the promiscuous AR fostered slower tumor growth than a poorly differentiated tumor lacking AR altogether.

3.3.2. Unchaperoned oncogenic behavior: AR-E255K

The AR NTD has been less examined for somatic mutation, but in the eleven human samples we studied 19 mutations recurred in the NTD and 14 of these fell into known functional motifs. The most highly conserved region of the AR NTD encodes a domain that interacts with CHIP (*C*OOH-terminus of *H*SP70-*I*nteracting *P*rotein), an E3-ubiquitin ligase that controls steady-state levels of AR by promoting its degradation (He et al., 2004). *Ar* mutations in this region have been identified in both mouse and human prostate tumors and may abrogate cell checks on AR activity. Importantly, the mouse mutant, AR-E231G, is sufficient for oncogenesis as a prostate-targeted transgene (Han et al., 2005). A similar human mutant, AR-E255K, from a flutamide-treated patient prolongs the half-life of AR in both the presence and absence of ligand (Steinkamp et al., 2009). Moreover, a substantial proportion of AR-E255K localizes to the nucleus in the absence of hormone. CHIP interaction domain mutants are not only unfettered from ligand control but also exhibit increased and differential transactivation (Buchanan et al., 2009; Steinkamp et al., 2009). For AR-E255K this is more notable in benign RWPE cells than in malignant PC-3 cells and varies with target promoter; differences in specificity as well as activity could reflect differences in the array of host coregulators. Varied transactivation by mutant ARs demonstrate how they not only drive persistent gene expression in the face of androgen ablation but that they may do so differentially, promoting distinct pathways of tumor progression that may differ in selective value dependent on disease stage.

3.3.3. Avenues of differential gene expression: AR-W435L, AR-R753Q

Mutations in almost any receptor domain may influence target gene selection via conformational changes that impact recognition or affinity for the response element or that alter intra- or intermolecular interactions. Two mutations from our studies exemplify the multiple ways transcriptional selectivity may be affected. AR-W435L, found in two antiandrogen-treated patients, alters the WxxLF motif that plays a role in AR's critical N-C interaction. While this motif is secondary to the more N-terminal FxxLF motif essential for ligand-dependent activity, WxxLF may be more important in ligand-independent activation (Dehm et al., 2007). W435L changes the motif to LxxLF, which bears greater similarity to the LxxLL interaction motif of coactivators. The effect of this is to strengthen N-C interaction, as demonstrated in a mammalian two-hybrid assay, and to confer stronger transactivation of a reporter gene (Steinkamp et al., 2009). Interestingly, AR-W435L activity varies with the host cell and target promoter tested, suggesting differential promoter recognition as well as broadly increased AR activity may have selective value and may change over the course of disease.

Some AR alterations that arise in prostate cancer are presumed to be null mutations, particularly if they were previously identified in AIS. Yet some have partial or altered function that could be significant in cancer. AR-R753Q is especially intriguing since as a germline mutation it underlies rat testicular feminization as well as cases of human AIS. The molecular basis for this is reduced androgen binding capacity resulting in impaired N-C interaction. In transfection into prostate cells, AR-R753Q proves to be as potent as wild type AR at activating a subset of promoters. AR-R753Q fails to activate selective AREs that bind AR with greater specificity but show weak activation (O'Mahony et al., 2008). A similar inability to activate AR selective elements occurs in a mouse with an AR DBD mutation that leads to reduced fertility (Schauwaers et al., 2007). Thus although germline AR-R753Q cannot activate genes critical for male development, somatic AR-R753Q may induce a subset of genes, perhaps those involved in oncogenic rather than differentiative pathways. Numerous other mutations may produce a similar

loss of function in the context of development but a gain of function in the context of cancer.

3.3.4. Alternative splicing as a path of resistance: AR23

Variant proteins that arise from alternative splicing (distinct from actual mutation) are widespread in cancer (David and Manley, 2010) and may be particularly significant in CRPC where constitutive ARs can be produced from misspliced transcripts that omit the LBD (Dehm et al., 2008; Hu et al., 2009). The mechanism by which these variants arise is unclear, but the fact that their occurrence increases rapidly when androgen is withdrawn and recedes when hormone is readministered suggests AR may be involved, directly or indirectly, in processing its own transcript. AR, like other steroid receptors, is known to participate in splicing (Auboeuf et al., 2002; Dong et al., 2007) and disease conditions or aberrant ARs may foster aberrant splicing events (Yu et al., 2009). Many misspliced ARs terminate prematurely, destroying their ability to bind ligand and freeing them from cytoplasmic tethering by HSP90, thus permitting their localization to the nucleus where they are constitutively active. These variant forms are usually a small fraction of the total *Ar* mRNA in the cell and it remains unclear whether they provide a major means of treatment resistance (Watson et al., 2010; Li et al., 2011).

A distinct misspliced *Ar* called AR23 differs from the constitutive variants in lacking intrinsic activity but promoting full-length AR function. We found this variant in the majority of antiandrogen-treated patient samples (Steinkamp et al., 2009). Use of a cryptic splice site in intron 2 inserts 69 bps in frame to create a 23 amino acid extension between the two zinc fingers of the DBD. AR23 was previously identified in an AIS patient due to a mutation upstream of exon 3 and also in a bicalutamide-treated prostate cancer patient (Bruggenwirth et al., 1997; Jagla et al., 2007). Beyond being incapable of binding DNA, AR23 forms cytoplasmic speckles in response to hormone. While this receptor cannot participate directly in gene regulation, cotransfection of AR23 with wild type AR leads to greater activation of a reporter gene, even in the presence of antagonist. This action is not specific to AR since some other transcription factors are also enhanced in their activity in the presence of AR23. AR23 likely exists in an unfolded state, as suggested by its cytoplasmic aggregation, which may compromise the cell chaperone system critical for normal protein activity. In this manner AR23 may sustain wild type AR activity in the presence of antagonists, as supported by its prevalence in treated but not untreated patients.

4. Concluding remarks

The genomic era has added significant depth to our understanding of prostate cancer. Some discoveries are remarkable, such as the unanticipated and broad role of *TMPRRS2-ERG* gene fusions. These findings only serve to substantiate AR as a central player in all stages of disease and, as yet, the key target in therapy. Studies with genetically engineered mice allow functional analyses not possible in man, using homogeneous genetic backgrounds and uniform environments to highlight distinct disease modalities. Results from our lab and others confirm the relevance of *Ar* genetic variation to disease progression, and derive novel insights into mechanisms of treatment resistance from specific variations.

In mice, differences in AR Q tract length exert measurable effects on disease parameters including development of PIN and rate of cancer progression, both in the presence and absence of hormone. While Q tract length is not by itself a risk factor for prostate cancer in man, it contributes to variation in the androgen axis, in which many genes conspire to influence hormone sensitivity. Q tract lengths at the extremes of the normal range may have more detectable effects when hormone levels are changing, as occurs in development and aging, or clinically, as when hormone is replaced in hypogonadal men or ablated in prostate cancer treatment. Individual variation in efficacy of new drugs that more effectively inhibit androgen synthesis (e.g., abiraterone) may reveal an influence of AR Q tract length.

Somatic *Ar* mutations are generally low in frequency but provide valuable information about mechanisms of treatment resistance. Myriad mutations affect multiple distinct functions (specificity of hormone and DNA binding, coactivator and chaperone interactions, nuclear localization), with downstream gene expression programs varying with the receptor form. AR's enormous plasticity in evading treatment is similar to the complexity p53 demonstrates whereby many different mutations drive transition from tumor suppressor to oncogene. The mutability of AR is but one factor making it unlikely that single agents will universally abolish activity. Promising new drugs such as MDV3100 and EPI-001 provide new environments for selection (Sadar, 2011); cases of resistance may reveal additional novel AR alterations. Moreover, improved anti-AR therapies may impair differentiation and therefore be ineffective in redirecting cell growth in certain instances. The mutant ARs direct attention to upstream and downstream interacting partners that may provide new ways to combat disease in general or to fight resistance in specific cases. The broad landscape of low level *Ar* mutation suggests AR is more than a passenger, but not often a driver. Nevertheless the difficulty of silencing AR in prostate cancer suggests it acts as a backseat driver.

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Predicting Response to Hormonal Therapy and Survival in Men with Hormone Sensitive Metastatic Prostate Cancer

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Abstract

Androgen deprivation is the cornerstone of the management of metastatic prostate cancer. Despite several decades of clinical experience with this therapy there are no standard predictive biomarkers for response. Although several candidate genetic, hormonal, inflammatory, biochemical, metabolic biomarkers have been suggested as potential predictors of response and outcome, none has been prospectively validated nor has proven clinical utility to date. There is significant heterogeneity in the depth and duration of hormonal response and in the natural history of advanced disease; therefore to better optimize/individualize therapy and for future development, identification of biomarkers is critical. This review summarizes the current data on the role of several candidate biomarkers that have been evaluated in the advanced/metastatic disease setting.

Keywords: prostate cancer, androgen deprivation therapy (ADT), hormonal therapy, prostate-specific antigen (PSA), prognostic biomarkers, predictive biomarkers, androgens, androgen receptor (AR)

Vitae

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Dr. Hussain is a graduate of the Baghdad University Medical School and completed her internal medicine residency and medical oncology fellowship at Wayne State University. Dr. Hussain is a Professor of Medicine and Urology and the Associate Director for Clinical Research at the University of Michigan Comprehensive Cancer Center (UMCC) and a Co-leader of its Prostate Cancer Program. She is the Associate Chief for Clinical Research at the Division of Hematology/ Oncology, Department of Internal Medicine. Dr. Hussain is an internationally recognized clinical researcher and expert in genitourinary malignancies particularly bladder and prostate cancers. Her research is focused on novel therapeutic interventions and designs and she is funded by several peer reviewed grants. The author of over 165 scholarly articles and book chapters, Dr. Hussain has served on the editorial boards for several national and international specialty publications. Nationally, Dr. Hussain serves as the co-chair for the Prostate Cancer sub-committee of SWOG GU committee. She is a member of several federal and national scientific review panels including NCI, DOD, ASCO, and AUA. Dr. Hussain is the 2012 chair elect of the Integration Panel of US Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program. She has served as chair of the Education Committee of ASCO and was the Chair for 2011 ASCO Genitourinary Cancer Symposium Program Committee. She has served as a member and the Chair for the FDA Oncology Drug Advisory Committee (ODAC).

1. Introduction

In 1941, Charles Huggins and Clarence Hodges demonstrated that castration halted the progression of prostate cancer implying that testosterone was the driver of prostate cancer cell proliferation and survival. Since then, androgen deprivation therapy (ADT) (castration) has become the cornerstone of the systemic treatment of advanced prostate cancer. Although this therapy is associated with a high antitumor effect in approximately 90% of patients, its role in advanced disease is palliative [1]. Despite 70 years of ADT, progress to date has been limited to the development of different modalities to mediate androgen deprivation, and unlike breast cancer there are no standard assays with clinical utility assessing the androgen receptor. Considering the heterogeneity in the depth and duration of hormonal response and in the natural history of advanced prostate cancer, biomarkers are clearly needed not only to better personalize therapy but also to focus and expedite new therapy development for those who are not destined to respond well to standard ADT.

Biomarkers can serve a variety of purposes. A prognostic biomarker is one that correlates with outcome, independent of treatment effects; examples include performance status, stage, Gleason's score and PSA. A predictive biomarker provides information about the probability of benefit or toxicity from a specific therapy. Examples include the expression of estrogen receptor (ER) as predictor of response to hormonal therapy in breast cancer, the mutation of KRAS as negative predictor of response to anti-EGFR therapy in colorectal cancer and NSCLC, or the V600E mutation of BRAF as predictor of response to BRAF inhibitor in melanoma.

Despite the extensive experience with prostate cancer in general and with hormone therapy in advanced stage disease, to date there are neither predictive response biomarkers to hormone therapy nor surrogate biomarkers for survival. Candidate biomarkers are often derived from retrospective multivariate analysis of clinical studies, in which such markers are correlated with outcome**.** It is often difficult to discern the prognostic *vs* predictive value of a biomarker, especially when there is no appropriate statistical design or a comparator control group in a clinical trial. In this review, we discuss the role and current data of several candidate predictive or prognostic biomarkers in patients with advanced/metastatic prostate cancer.

2. Potential Biomarkers of response to hormonal therapy (Table 1)

2.1. Androgens

Early studies attempted to investigate the role of hormone levels in determining response to androgen deprivation therapy. Geller et al measured dihydrotestosterone (DHT) concentration in prostatic tissue from patients with recurrent advanced prostatic cancer after estrogen therapy or castration with or without estrogen therapy [2]. A small proportion of patients had DHT level above the expected castration level. The authors suggested that

patients who are castrated or treated with estrogen should have plasma testosterone and tissue DHT measurement at the time of relapse; if plasma and tissue DHT are at castrate level, further hormonal therapy would not be indicated. They also suggested adrenocortical suppression if plasma testosterone is at castrate level and tissue DHT is elevated, implying that adrenal androgen production can contribute to high tissue DHT concentration. They proposed that if testosterone is above castrate level, titration with hormonal therapy should be attempted while plasma testosterone is monitored to achieve castrate level.

The potential role of testosterone as a biomarker of response to ADT was investigated in a retrospective review of 129 patients with hormone-naïve metastatic to bone-only disease treated with 3 months of goserelin (LHRH agonist) [3]. Testosterone and PSA were measured every 3 months. Statistical analysis showed that the risk of death directly correlated with Gleason score ($p<0.01$), 6-month PSA ($p<0.01$), and 6-month serum testosterone level ($p<0.05$). The authors encouraged the achievement of optimal reduction of testosterone level with treatment.

The role of adrenal androgens has been a focus for therapy. Data from a Japanese study in castration-resistant prostate cancer patients treated with combined androgen blockade using flutamide as a second-line anti-androgen therapy Indicated that response rate and duration to flutamide was predicted by higher baseline androstenediol and lower dehydroepiandrosterone (DHEA) level, suggesting that adrenal androgen metabolites could contribute to the progression of prostate cancer [4]. Recent reports suggest that intra-prostatic/tumor concentrations of androgen hormones can be altered with ADT and might be a surrogate marker of tumor response to anti-androgens; however, data have been inconsistent [5, 6]. A caveat regarding the utility of androgen level as predictive biomarker relates to the variability within a patient over time and the different assays by separate labs.

2.2. Androgen receptor (AR)

2.2.1. AR expression

Trachtenberg et al studied whether pre-treatment prostatic AR measurement could have predictive value in 23 patients with metastatic prostatic cancer treated with hormonal therapy [7]. Cytosolic and nuclear prostatic AR contents from pre-treatment biopsies were measured. All patients had measurable AR levels in prostatic tissue and demonstrated objective improvement with hormonal therapy. There was a strong correlation between duration of response and survival ($p<0.01$). Neither total cellular nor cytosolic AR content was associated with response; however, nuclear AR content correlated with response duration and survival ($p<0.05$). The median duration of response (7.1 *vs* 17.3 months) and median survival (14.4 *vs* 24.7 months) was significantly shorter in patients with lower compared to higher level of nuclear receptor. This was the first report of an association between nuclear AR content and hormonal response and might underline the “driving” AR transcriptional role. A study of 62 patients with untreated metastatic prostate cancer who received ADT showed that high immunohistochemical (IHC) AR content is a favorable prognostic indicator [8]. In addition,

image IHC analysis of prostate cancer AR with a pattern/receptogram-oriented approach could accurately predict response to hormone therapy in patients with advanced stage, while AR heterogeneity was associated with poor prognosis in different studies [9-11].

The identification of discrete AR epitopes by color video image analysis was associated with prognosis [12]. A study using microarray-based profiling of isogenic prostate cancer xenograft models found that a modest increase in AR mRNA was the only change consistently associated with the development of resistance to anti-androgen therapy, underlining the significance of the disease setting and timing of assessment [13]. Furthermore, this study used cell models to demonstrate that increased AR level is sufficient to confer anti-androgen resistance, in part by converting antagonists to weak agonists.

Another study measured multiple biochemical variables and determined Gleason grade in prostatic biopsies obtained from 16 patients before castration or initiation of estrogen therapy [14]. Biochemical variables included 3 enzymes involved in androgen metabolism and 3 hydrolytic enzymes, AR content, tissue testosterone and DHT content. A group of 7 patients had mean response duration of 7.7 months, relapsed and died of the disease, while 9 patients had mean response duration of 18.6 months and were still responding at the time of the report. The 2 groups could not be distinguished by Gleason grade, single enzymatic activities or tissue androgen content. However, AR level was statistically different between the groups ($p<0.05$) but with considerable overlap of individual patients. The authors developed an index based on multiple enzymatic activities, which separated the groups better than any single variable alone ($p<0.02$). When salt extractable nuclear AR was included in the numerator of this index, the groups were separated almost completely, implying that the measurement of multiple biochemical variables may be a useful predictive marker.

As recently presented, PSA response to MDV3100, a novel AR inhibitor tested in patients with castration-resistant prostate cancer, was associated with unchanged or reduced AR expression ($p=0.031$). Higher baseline bone marrow and plasma testosterone level were also associated with PSA response ($p<0.05$) [15]. Studies noted above indicate that AR expression and function can change through tumor progression and may be affected by specific treatment agents with different mechanisms of action, such as the novel anti-androgens. Therefore, the role of AR expression must be interpreted relative to the “context” of disease stage, previous and current treatment.

2.2.2. AR gene amplification

Koivisto et al suggested that hormone-refractory prostate tumors are genetically complex and show intra-tumor genetic heterogeneity [16]. Increased copy number of chromosome X and AR gene amplification may confer proliferative advantage during ADT and contribute to recurrence. They later reported that AR gene amplification was more common in prostate tumors that initially responded well to ADT, and those with response duration >12 months

[17]. Tumors with earlier local relapse or no initial response did not contain AR gene amplification. The median survival after local recurrence was twice as long for tumors with AR gene amplification (p=0.03) as for tumors with single copy AR. However, 28% of locally recurrent tumors contained AR gene amplification compared to none of the untreated primary tumors, implying that ADT local failure may be caused by a clonal expansion of tumor cells that could continue androgen-dependent growth, despite low concentrations of serum androgens. It was concluded that AR gene amplification and wild-type over-expression may play a role in ADT failure.

2.2.3. AR gene mutations

Taplin et al reported AR mutations in 5 of 10 distant metastases from patients with ADT failure, suggesting that AR gene mutations can play a role in the metastatic progression of prostate cancer [18]. There have been data on the potential role of AR mutations in the development of androgen independence after ADT [19, 20]. Such mutations occur in distinct portions of the AR gene encoding the unstructured amino terminus (NTD), ligand-binding domain (LBD) or DNA-binding domain (DBD), and could affect ligand specificity, transcriptional activity, or mediate androgen-independent functions [21]. Mutated AR could exploit different mechanisms to evade hormonal deprivation, while many of those mutations could be selected after specific hormonal manipulation [22]. An example of mutated AR includes AR-V716M variant that creates a permissive, “promiscuous” receptor that can respond to a variety of hormone agonists and antagonists [23]. AR-E255K is another AR variant that localizes to the nucleus in the absence of androgen, promoting gene expression that could result in tumor progression [22]. AR-W435L mutation has been found in 2 patients treated with anti-androgen therapy and may promote conformational changes that impact inter-/intra-molecular interactions that influence recognition or affinity for gene response elements [22]. Some AR mutations may behave as “loss of function” in the context of development but as “gain of function” in the context of malignant transformation and treatment. AR variants can also arise from alternative splicing; their frequency increases rapidly with ADT and recedes upon hormone re-administration [24, 25]. Their role in treatment resistance may be significant since several of these AR forms are active in the absence of ligand.

2.2.4. AR polyQ tract gene polymorphisms

The N-terminal polyQ tract, encoded by a CAG repeat has been inversely correlated with AR transcriptional strength [26]. Sensitivity to Q tract length difference within a ‘‘normal’’ range (9–37 CAG repeats) is probably due to effects on overall AR structure rather than to accessory protein interactions at that site [27]. A clear association between Q tract length and prostate cancer remains controversial. However, results from genetically engineered mice support further investigation of the role that AR Q tract variation may play in late stage prostate cancer and provide a unique experimental model in which to define downstream

events that may predict response to therapy [20]. For example, mice bearing tumors with a long CAG repeat (AR48Q polymorphism) fail to benefit from androgen ablation [20].

2.3. Other genetic polymorphisms

2.3.1. Luteinizing hormone-releasing hormone receptor (LHRHR), luteinizing hormone receptor (LHR), steroid hormone binding globulin (SHBG)

A recent study of patients with advanced or high-grade prostate cancer from different ethnic backgrounds reported a significant association between disease-specific survival and genetic variation in LHRHR, LHR, AR and SHBG [28]. Hazard ratios (HR) for carriers *vs* non-carriers of the LHR312 minor allele were 1.63 (95% CI 1.08-2.45) among all cases and 2.04 (95%CI 1.23-3.39) for high-grade cases. The LHRHR16 minor allele was rare in African Americans; in Caucasians (HR 1.90, 95%CI 1.15-3.13) it did not correlate with grade. The SHGB356 minor allele was associated with survival only among high-grade tumors (HR 2.38, 95%CI 1.18-4.81). It was concluded that genetic variations in the LHRHR, LHR, AR and SHGB genes were associated with outcome and merit further analysis to define their role as prognostic and predictive biomarkers; however treatment information was not provided.

The potential impact of genetic polymorphisms in LHR on the response to ADT was evaluated in 50 patients with prostate cancer [29]. Twenty-nine patients were treated for primary metastatic disease, 18 for biochemical recurrence and 12 for biochemical recurrence and radiographic metastases. In the biochemical recurrence subset, the presence of minor alleles in the LHR genotype was associated with shorter time to castration resistance; 39 months in patients with no minor alleles, 23 months in patients with 1 minor allele and 16 months in patients with 2 minor alleles ($p<0.05$). The median testosterone level during ADT was higher for patients carrying minor alleles; 7 ng/dL for patients with no minor alleles *vs* 22.5 ng/dL for patients with 1 or 2 minor alleles ($p=0.07$) with median peak testosterone level of 19 ng/dL *vs* 43 ng/dL, respectively ($p=0.01$). It was concluded that LHR genetic variation correlated with time to castration resistance and depth of castration, supporting the design of further studies to define its value as a prognostic and predictive marker.

2.3.2. Androgen biosynthesis and metabolism

Germline genetic variation in the androgen metabolic pathway was evaluated as a predictor of hormonal therapy efficacy [30]. 529 men with advanced prostate cancer treated with ADT were genotyped for 129 DNA polymorphisms of 20 genes involved in androgen metabolism. Three polymorphisms in separate genes (CYP19A1, HSD3B1, and HSD17B4) correlated with longer time to progression (TTP) during therapy ($p<0.01$), which was confirmed in multivariate analysis. Patients with >1 of these polymorphisms responded better to therapy than patients with 0-1 polymorphism ($p<0.0001$). This report suggested that genotyping of specific genetic loci might improve ADT response prediction, underlining the significance of pharmacogenomics.

Another recent study attempted to determine the predictive value of a genetic polymorphism in the testosterone transporter gene relating to ADT response duration [31]. The same group had previously shown that a polymorphism in SLCO1B3 gene increases testosterone transport into cells, and that the presence of at least 1 of the more common T allele at the 334 T>G polymorphism in this gene correlates with shorter survival. The group examined the association between this SLCO1B3 polymorphism and time to treatment resistance (androgen independence) in 68 patients with metastatic or biochemically recurrent prostate cancer. Men with the T allele tended to have shorter time to androgen independence in both patient cohorts (p=0.11, p=0.18). Combining the cohorts and stratifying by stage provided a significantly shorter time to androgen independence with the presence of the T allele (p=0.048). Another study suggested that the presence of the T allele is associated with rapid testosterone uptake by cells and shorter duration of response to ketoconazole, a 17 lyase inhibitor used as secondary hormonal therapy [32].

The association of germline variation in genes regulating androgen biosynthesis and metabolism pathways with response duration in prostate cancer patients receiving ADT was evaluated [33]. Overall, 747 single nucleotide polymorphisms (SNPs) from 84 genes in 304 patients with advanced prostate cancer who progressed on ADT were tested. The median patient age at ADT failure was 72 and the overall median time to ADT failure was 3.21 years. At the gene level, *TRMT11* (tRNA methyltransferase 11 homologue) exhibited the strongest association with time to ADT failure (p<0.0008); 2 out of 4 *TRMT11* tag SNPs were associated with time to ADT failure. The first was the intronic SNP rs1268121 (A>G) with a positive association of median time to ADT failure with the number of variant alleles (p=0.023): 3.1 years for 0, 4.1 years for 1, and 5.9 years for 2 variant alleles. The second was the SNP rs6900796 (A>G) in the 3' untranscribed region with a median time to ADT failure of 2.6 years for 0, 2.5 years for 1, and 3.8 years for 2 variant alleles (p=0.023). Four additional genes correlated significantly with ADT response (*LOC390956*, *PRMT3*, *SLC7A6OS*, and *WBSCR22)*, but all 4 genes had a false discovery rate >0.95.

2.3.3. Hormone response elements and genetic risk variants

The prognostic role of 49 SNPs in the estrogen response element (ERE) of particular genes was evaluated using a genome-wide database in 601 men with advanced prostate cancer treated with ADT [34]. Based on multiple hypotheses testing, BNC2 rs16934641 was found to be associated with disease progression; TACC2 rs3763763 was associated with prostate cancer-specific mortality, while ALPK1 rs2051778 and TACC2 rs3763763 were associated with all-cause mortality. The statistical significance remained in multivariate analysis with known clinicopathological prognostic factors. A combined genotype effect on all-cause mortality was observed when ALPK1 rs2051778 and TACC2 rs3763763 were analyzed in combination. Patients with a greater number of unfavorable genotypes had shorter time to all-cause mortality during ADT (p<0.001). This group also investigated 55 common SNPs in

the genome-wide *in silico*-predicted androgen receptor elements (AREs) in the same cohort [35]. In univariate analysis, 2, 5 and 4 SNPs were associated with disease progression, prostate cancer-specific mortality, and all-cause mortality, respectively. In multivariate analysis after adjusting for known prognostic markers, ARRDC3 rs2939244, FLT1 rs9508016, and SKAP1 rs6504145 remained significant predictors for prostate cancer-specific mortality, while FBXO32 rs7830622 and FLT1 rs9508016 remained significant predictors for all-cause mortality. Strong combined genotype effects on outcome were also observed ($p<0.001$). Data have also indicated that PSA gene ARE1 -158 G/A polymorphism had no effect on PSA promoter activity *in vitro* and no association with PSA level in Japanese men; however, data suggested that ARE1 GG genotype may confer sensitivity to ADT, implying its potential value as predictive biomarker [36].

The prognostic value of 19 prostate cancer susceptibility variants in the previously described patient cohort was also investigated [37]. Two polymorphisms, rs16901979 and rs7931342, were significantly associated with prostate cancer-specific mortality ($p=0.005$, $p=0.038$, respectively), and rs16901979 was associated with all-cause mortality ($p=0.003$) after ADT. Although the effect of rs7931342 was attenuated after controlling for known clinical prognostic markers, rs16901979 remained a significant predictor of outcome after ADT ($p=0.002$). The addition of the rs16901979 status in the current clinical staging system enhanced the risk prediction of outcome particularly in the high-risk patients with distant metastasis ($p<0.017$). These results implied that incorporation of ERE, AREs and prostate cancer risk variants SNPs into predictive models might improve outcome prediction in patients with prostate cancer receiving ADT.

Germline AR-CAG repeat lengths had no significant correlation with TTP or overall survival in a group of 480 patients with recurrent or metastatic prostate cancer treated on ADT [38]. Longer AR-CAG repeat lengths (>23 *vs* ≤23) showed a non–significant trend towards longer TTP in metastatic disease. In contrast to the genetically homogeneous mice mentioned above [20, 39], in man the AR-CAG repeat length is not, by itself, informative for response to therapy. Future studies may reveal whether it has value in concert with other markers.

2.4. TMPRSS2:ERG fusion

Genetic alterations of the androgen-regulated gene TMPRSS2 and ETS transcription factor family members ERG, ETV1, ETV4, ETV5 have been identified as a common molecular event in prostate cancer. TMPRSS2:ERG gene fusions are the dominant molecular subtype, present in almost half of prostate tumors [40]. More than 90% of ERG-over-expressing prostate cancers harbour TMPRSS2:ERG fusions [41]. ERG over-expression could contribute to androgen-independence potentially due to AR signaling disruption, while TMPRSS2:ERG expression is restored in castration-resistant setting and may contribute to progression [42, 43]. The key role of the fusion might be bypassed by androgen-independent expression of wild-type ETS factors in late-stage prostate cancer [44]. It was shown that

histone deacetylase inhibitors and AR inhibitor flutamide can cause AR retention in cytoplasm, indicating androgen signalling inhibition, sustaining that this combination could be effective against TMPRSS2:ERG fusion-positive prostate cancer *in vitro* [45].

Preliminary data on the predictive value of TMPRSS2:ERG fusion has been contradictory. In one report using RT-PCR in CTC as a biomarker of sensitivity to abiraterone in 41 men with chemotherapy-treated castrate-resistant prostate cancer, TMPRSS2:ERG fusion was present in 15 patients, with a median baseline CTC of 17 [46]. PSA decline ≥50% was noted in 7 patients with and 10 patients without the fusion. TMPRSS2:ERG fusion status did not predict PSA response or other outcome. Another study also found no association between TMPRSS2:ERG fusion and outcome in prostate cancer patients primarily treated with hormone therapy, suggesting that it may not implicate hormone dependence [47]. In that study, IHC expression of protein SPINK1 was found in approximately 10% of tumors and was associated with significantly shorter progression-free survival. On the other hand, significant association between ERG gene rearrangements in CTC and the magnitude of PSA response ($p=0.007$) were reported in another study of patients with therapy-naive or castration-resistant prostate cancer treated with abiraterone [48]. In this cohort, PSA response was associated with decreased CTC and longer survival. In a cohort of 150 patients treated with radical prostatectomy, response to adjuvant ADT was associated with ERG fusion, with more significant treatment effect in ERG fusion-positive tumors [49]. Data indicated that patients treated with neo-adjuvant ADT who fail to suppress the expression of the TMPRSS2:ERG fusion have a higher risk of recurrence [50]. It is notable that a mechanistic rationale for poly-ADP-ribose polymerase (PARP) inhibition in ETS gene fusion-positive prostate cancer was recently reported [51].

2.5. Other molecular biomarkers

AR interacts in a comprehensive and dynamic manner with several co-regulators within the prostate cell microenvironment, forming an 'AR core pathway'. A recent genomic analysis identified alterations in the components of this pathway in all examined samples from prostate cancer metastasis [52]. Dissection of the molecular interactions between AR and transcriptional co-regulators as well as neighbouring signaling pathways could identify additional mechanisms and thus candidate biomarkers of ADT resistance. For example, over-expression of 2 transcription factors, transcriptional intermediary factor 2 (TIF2) and steroid receptor co-activator 1 (SRC1) can increase AR transactivation at physiological concentrations of adrenal androgen and can provide a mechanism for ADT resistance [53]. TIF2 gene amplification and protein over-expression has been associated with prostate cancer progression [52, 54]. SRC3 (AIB1) is another important regulator of prostate cancer proliferation, apoptosis and survival; SRC3 over-expression has been reported in prostate cancer patients [55]. Additionally, *in vitro* data indicated that up-regulation of survivin via IGF-1 signaling confers resistance to flutamide in prostate cancer cells [56]. Similarly, it was

shown that over-expression of bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo* [57]. A recent comprehensive review commented on the potential role of cytokines, such as interleukins; growth factors, such as Epidermal Growth Factor (EGF); intracellular kinase signaling, such as MAPK, PI3K/Akt/m-TOR, JAK/STAT, protein kinase A, and transcription factors, such as NF-kB in the development of ADT resistance [58]. It has been reported that components of the PI3K signaling pathway were genetically altered in all samples derived from prostate cancer metastasis that were examined by integrative genomic profiling [52].

At the post-genomic era, refined molecular analysis and sophisticated biochemical, genomic and proteomic platforms can contribute to the identification of novel predictive biomarkers [59]. Tumor tissue and CTC sequencing, gene expression and micro-RNA profiling techniques could reveal the potential predictive role of specific genomic, transcriptomic and micro-RNA signatures. For example, gene expression profiles of ADT-resistant tumors demonstrated that gene expression changes detected initially during ADT were no longer present suggesting reactivation of androgen and AR pathway in the absence of exogenous hormone [60]. A study using cDNA microarrays containing 1176 known genes demonstrated that 34 genes are up-regulated and 8 genes are down-regulated in androgen-independent cells [61]. Northern blot analysis did confirm the differences identified by microarrays in several candidate genes, such as c-myc, c-myc purine-binding transcription factor (PuF), macrophage migration inhibitory factor (MIF), macrophage inhibitory cytokine-1 (MIC-1), lactate dehydrogenase-A (LDH-A), guanine nucleotide-binding protein Gi, alpha-1 subunit (NBP), cyclin dependent kinase-2 (CDK-2), prostate-specific membrane antigen (PSMA), cyclin H (CCNH), 60S ribosomal protein L10 (RPL10), 60S ribosomal protein L32 (RPL32), and 40S ribosomal protein S16 (RPS16). These differentially-regulated genes correlated with human prostate cancer progression.

2.6. Novel imaging

Novel imaging modalities, such as diffusion-weighted and dynamic contrast-enhanced MRI, have also been investigated in the preclinical setting showing that the combination of early changes in several functional MRI parameters could provide additional information about therapy response and thus may help identify early non-responding patients, allowing consideration for alternative strategies [62]. Non-invasive measurement of AR signaling with a positron-emitting radiopharmaceutical targeting PSMA could be another useful tool guiding AR activity alterations during ADT [63].

3. Biomarkers of outcome (Table 2)

3.1. Prostate-specific antigen (PSA, Table 3)

The most frequently evaluated biomarker of response to hormonal therapy has been PSA level and kinetics. A multicenter Belgian study of 546 patients reported that PSA <4 ng/ml after 3 or 6 months of hormone therapy along with initial tumor grade, stage, and performance status are the most significant factors predictive of progression-free survival of hormonal treatment in patients with advanced prostatic cancer [64]. On the other hand, results from a meta-analysis, utilizing data from 3 randomized clinical trials suggested that PSA could not be statistically validated as a surrogate for overall survival; however, PSA ≤4ng/ml was a potent prognostic factor for survival [65]. This meta-analysis was criticized based on the fact that patients were not treated uniformly, PSA was not monitored in a similar way, and it was not clear whether the same PSA assay or calibrated assays were implemented in different trials.

Baseline prognostic factors in patients with metastatic prostate cancer treated with combined androgen blockade *vs* monotherapy were explored in SWOG-8894 (a phase III trial of orchiectomy with or without flutamide) [66]. Significant baseline factors were identified, but they did not accurately predict survival of individual patients. Only 13% of patients who survived >=10 years were predicted by the model *vs* 98% of men who died within 5 years (Table 3). Data from SWOG-9346, a randomized trial of intermittent *vs* continuous hormone therapy, were used to assess whether absolute PSA after ADT is prognostic for survival in 1,345 patients with metastatic prostate cancer and a baseline PSA ≥5ng/ml [67]. After 7 months of induction ADT, 1,134 patients achieved a PSA ≤4.0 ng/ml on months 6 and 7 and were randomly assigned to continuous *vs* intermittent ADT on month 8. Men with PSA 0.2-4ng/ml had less than one third the risk of death compared to those with PSA >4ng/mL ($p<0.001$). Men with PSA ≤0.2ng/ml had less than one fifth the risk of death compared to men with PSA >4ng/mL ($p<0.001$), and significantly longer survival *vs* those with PSA 0.2-4ng/ml ($p<0.001$). The median survival was 13 months for patients with PSA >4ng/ml, 44 months for PSA 0.2-4ng/ml, and 75 months for PSA ≤0.2ng/ml. A landmark meta-analysis reported that PSA progression, defined as an increase of at least 25% than PSA level at 7 months and an absolute increase of at least 2 or 5 ng/ml, can predict survival and may be a suitable endpoint for phase II studies in the appropriate settings [68].

A few studies have associated undetectable PSA nadir with durable response to ADT [69-71]. Other studies showed that shorter time to PSA nadir after ADT initiation was associated with shorter survival, implying that a rapid initial response might indicate more aggressive disease [72, 73]. Another study associated PSA at ADT initiation with TTP in patients without metastasis; Gleason score predicted TTP in patients with metastasis [74]. PSA nadir and time to nadir were evaluated as predictors of survival in patients with bone metastasis [75]. Survival was longer in patients with lower PSA nadir. Patients with longer time to PSA nadir (>9 months) had longer survival in both lower and higher PSA nadir subgroups. A 20-year retrospective review of patients receiving primary and salvage ADT associated PSA before ADT with overall and disease-specific survival [76]. The clinical significance of PSA half-time

(time for PSA to reach half of its baseline level during ADT) and doubling time after PSA nadir as predictors of response to ADT were examined in patients with metastatic disease [77]. Baseline and PSA nadir did not differ in patients with short (≤1 month) *vs* long (>1 month) PSA half-time. Patients with short PSA half-time had higher Gleason score, shorter PSA nadir duration and shorter cancer-specific survival. Patients with short (≤6 months) *vs* long (>6 months) PSA doubling time after PSA nadir did not differ in baseline PSA, PSA nadir, Gleason score and PSA half-time (Table 3).

3.2. Inflammatory markers (C-reactive protein, Interleukin-6)

It has been argued that cancer progression depends on a complex interaction between the tumor and the host inflammatory response and that the latter may have prognostic value in cancer patients. C-reactive protein (CRP) is a marker of systemic inflammatory response. It has been suggested that CRP could predict survival in patients with urological cancers, including prostate cancer, and that the incorporation of CRP into prognostic models for urological cancers improves the models' predictive accuracy [78]. The prognostic value of CRP was examined in 62 patients with metastatic prostate cancer receiving ADT [79]. On both univariate and multivariate survival analysis, CRP and PSA were significant predictors of cancer-specific survival. PSA was significantly correlated with CRP ($r(s)=0.46$, $p<0.001$). The results suggested that elevated CRP could predict poor outcome, independent of PSA, in patients with metastatic prostate cancer on ADT.

Interleukin-6 (IL-6) is a cytokine that may play a role in prostate cell regulation, prostate cancer development and progression [80, 81]. It can act as a growth signal in benign and malignant prostate cells. IL-6 and its receptors levels are increased during prostate cancer initiation and progression, and have been associated with poor prognosis [82, 83]. This is pertinent considering the cross-talk between IL-6- and AR- signaling [84]. However, differences among studies may be related to the fact that blood IL-6 levels may not reflect local concentrations in the tumor microenvironment [85]. In addition, host factors, like obesity and inflammation, may influence IL-6 levels. The predictive role of IL-6 in patients with metastatic prostate cancer on ADT has to be validated.

3.3. Metabolic markers: **Body Mass Index (BMI) and bone turnover markers**

A study showed that in men with androgen-dependent prostate cancer, higher BMI was associated with longer overall ($p<0.001$) and progression-free ($p=0.009$) survival, and higher likelihood to achieve PSA nadir <4ng/ml ($p=0.008$) [86]. In multivariate analysis adjusting for risk factors, higher BMI positively correlated with overall survival ($p<0.01$); overweight but not obese patients (BMI 27-29.9) had significantly better outcome *vs* normal-weight patients. A study of patients with local disease treated on ADT and irradiation correlated higher BMI independently and significantly with shorter time to PSA failure (HR 1.10, $p=0.026$) [87].

Markers of bone turnover were evaluated for their potential predictive value in patients with bone metastatic disease treated with ADT [88]**.** In multivariate analysis, 6-month markers

of bone turnover, which were all below the baseline median level (p=0.014), nadir PSA <0.2ng/ml (p=0.042) and lower metastatic volume at baseline (p=0.033) were associated with longer time to skeletal-related events. Bone turnover markers below the baseline median at 6 months and PSA nadir <0.2ng/ml were associated with longer time to castration resistance (p=0.026, p=0.058, respectively). The former was weakly associated with overall survival (p=0.092).

3.4. Circulating tumor cells (CTC)

The clinical utility of identifying and monitoring changes in CTC and CTC molecular determinants as surrogate biomarkers of response to ADT and survival has been evaluated in large trials of novel anti-androgens in castration resistant disease [89]. The phase III trial of abiraterone *vs* placebo prospectively assessed CTC as biomarker of survival in 1,195 patients with metastatic castrate-resistant disease [90]. CTC conversion using the standard definition for unfavorable (≥5) and favorable (<5) counts predicted overall survival at 4 weeks after treatment. CTC significantly reduced the treatment effect at all post-treatment time points (HR 0.74-0.97). A reduced model with CTC and LDH was developed and suggested for further evaluation. A study recently reported that molecular CTC alterations could serve as predictive markers of sensitivity and outcome [91]. In that study, sequencing coverage and polymorphism detection thresholds in heterogeneous cell population were confirmed. In another study, initial CTC value correlated with LDH and alkaline phosphatase, but was unrelated to PSA and testosterone [92]. In multivariate analysis, baseline CTC value retained independent predictive value. The cut-point that optimized test sensitivity and specificity was 3cells/7.5ml; baseline CTC value correlated with PSA nadir benchmarks. A retrospective study demonstrated that circulating microRNA-141 in CTC could predict clinical progression and treatment response compared to other biomarkers, such as PSA and LDH, in patients with metastatic prostate cancer [93].

Despite being very promising, this approach has limitations. CTC shed from primary and metastatic tumors are admixed with blood components and are very rare, with about 1 CTC/billion blood cells in patients with advanced cancer [94]. The identity of CTC, the mechanisms of shedding into the blood and the biological drivers of metastasis should be better characterized before using CTC as therapeutic targets and/or biomarkers. CTC isolation and characterization remain a major technological challenge; a standardized technique without inter-lab value variations is critical. In addition, it has been discussed that interaction between CTC and blood components, such as platelets, might alter the gene expression pattern of CTC, and thus their resemblance with the original tumor [95]. The prognostic/predictive value of CTC in prostate cancer has to be validated.

4. Conclusion

Several candidate biomarkers have been proposed as potential predictors of response to ADT; however, none has been prospectively validated nor has proven clinical utility to date. This is an area of unmet need and further studies are required to validate prospectively the value of genetic, epigenetic, hormonal, inflammatory, biochemical, metabolic and other molecular biomarkers in this setting. Prospective and robust validation of such biomarkers in appropriately designed and optimally executed clinical trials could result in better selection of patients who are predicted to respond to hormonal manipulation and spare the toxicity to those who are unlikely to benefit. Optimal sample storage, refined processing, quality control, and appropriate statistical designs are critical for a successful biomarker development endeavour.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1 Potential biomarkers of response to ADT

Serum and tissue androgens -testosterone, dihydrotestosterone, androstenediol, dehydroepiandrosterone
Androgen receptor -mRNA expression -protein expression -protein localization -gene amplification -gene polymorphisms(Q tract length) -gene mutations -splice variants
Other genetic polymorphisms -hormones (LHRH, LH) -receptors (LHRHR, LHR) -enzymes (androgen biosynthesis and metabolism, estrogen biosynthesis) -transporter and binding proteins (testosterone transporter, SHBG) -hormone response elements (AREs, EREs) -genetic risk (susceptibility) variants
Other molecular biomarkers -chromosomal alterations (TPMRSS2:ERG fusion gene) -gene expression profiling (microarrays, whole genome, exome and transcriptomic sequencing) -proteomics (mass-spectrometry) -micro-RNA and other non-coding RNA screening
Molecular imaging -DW-MRI, DCE-MRI -positron-emitting radiopharmaceuticals

Table 2 Potential predictors of survival (biomarkers of outcome)

Performance status
Co-morbidities [96]
Gleason grading score
Prostate specific antigen (PSA half-time, PSA nadir, time to PSA nadir, PSA doubling time after PSA nadir)
Inflammatory markers (C-reactive protein, IL-6)
Metabolic markers (body mass index, bone turnover markers)
Circulating tumor cells (CTC) / CTC genomic characteristics

Table 3 Studies evaluating the prognostic role of PSA along with other clinicopathological markers in men with advanced/metastatic prostate cancer treated with ADT

Ref(Year)	Number of patients	Prognostic factors (multivariate analysis)
66 (2003)	1286	minimal disease, bone pain, Gleason score, PSA
67 (2006)	1134	baseline PSA, Gleason score, performance status, bone pain
69 (2002)	153	Gleason score, PSA nadir
70 (2005)	185	PSA nadir, time to PSA nadir, bone scan findings, Gleason score
71 (2011)	650	PSA nadir, time to PSA nadir, metastatic disease, Gleason score
72 (2009)	179	time to PSA nadir, PSA nadir, Gleason score
73 (2011)	198	PSA nadir, time to PSA nadir/PSA nadir ratio
74 (2008)	553	Gleason score, metastatic disease, PSA at ADT initiation
75 (2011)	87	bone scan findings, PSA nadir, albumin, LDH
76 (2009)	548	age (diagnosis + ADT induction), stage, PSA at ADT induction
77 (2009)	131	PSA nadir, PSA half-time, PSA doubling time after PSA nadir