Glutamine tract length of human androgen receptors affects hormone-dependent and -independent prostate cancer in mice

Megan A. Albertelli1, Orla A. O’Mahony1, Michele Brogley1, Jeffrey Tosoian1, Mara Steinkamp1, Stephanie Daignault2, Kirk Wojno3 and Diane M. Robins1,*

1Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109-0618, USA, 2University of Michigan Cancer Center Biostatistics Core, University of Michigan Medical School, Ann Arbor, MI 48109-0473, USA and 3Department of Urology, University of Michigan Medical School, Ann Arbor, MI 48109-0946, USA

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The androgen receptor (AR) is involved in the initiation and progression of prostate cancer and its transition to androgen independence. Genetic variation in AR may contribute to disease risk and has been studied for a polymorphic N-terminal glutamine (Q) tract that shows population heterogeneity. While the length of this tract is known to affect AR in vitro, association with disease is complicated by genetic and environmental factors that have led to discordant epidemiological findings. To clarify the effect of Q tract polymorphism on prostate cancer, we created mice bearing humanized AR genes (h/mAr) varying in Q tract length. ARs with short Q tracts (12Q), which are transcriptionally more active, induce earlier disease in the transgene-induced TRAMP prostate cancer model than alleles with median (21Q) or long (48Q) tracts. Disease length varies within each genotype, with greater differentiation and AR expression in slower growing tumors. Remarkably, following androgen ablation, Q tract length has effects that are also allele-dependent and in directions opposite to those in hormone intact mice. Differences in AR activity conferred by Q tract length thus appear to direct distinct pathways of androgen-independent as well as androgen-dependent progression, and highlight substantial risk that may be associated with alterations in the androgen axis. This AR allelic series in humanized mice provides an experimental paradigm to dissect the role of AR in prostate cancer initiation and progression, to model response to treatment and to test therapies targeted specifically to the human AR.

INTRODUCTION

Androgens, acting via the androgen receptor (AR), are critical components in development, differentiation and disease of the prostate (1). This stems from AR’s function as a ligand-activated transcription factor that regulates the expression of numerous genes involved in cell proliferation and differentiation. Although many genetic and environmental factors impact prostate cancer, all prostate tumors initially require androgens for growth. Because of this dependence, prostate tumors regress when steroid signaling is abolished, either by surgical castration or by compounds that block androgen synthesis and antagonize AR action. Ultimately, however, most cancers recur in a hormone-refractory state resistant to further treatment (2). While these tumors are considered androgen-independent, AR levels often remain high and the AR signaling pathway appears to be intact, suggesting AR remains a factor in disease progression (3,4). This AR activity in the face of androgen ablation has been ascribed to multiple mechanisms, including AR gene (Ar) amplification, Ar gain-of-function mutations, ligand-independent activation of AR by growth factors and alterations in AR cofactors (5).

Due to the central role of AR in prostate cancer, subtle differences in receptor function or level may impact prostate...
cancer risk, disease progression or response to therapy. The most studied polymorphism in the human $AR$ (hAr) is variation in length of a CAG trinucleotide repeat encoding a polyglutamine (Q) tract within the N-terminal transactivation domain (NTD). In the general population, AR Q tract length ranges between 9 and 37 residues, with most tracts having 15–30 Qs (6). There is also a polymorphic polyglycine (G) tract, encoded by 8–18 GGN repeats. The G tract is implicated in pathology since expansion of the CAG repeat beyond 40 codons is associated with the late-onset neurodegenerative disease spinal and bulbar muscular atrophy (SBMA or Kennedy disease) (7).

In vitro studies demonstrate an inverse relationship between Q tract length and transcriptional activation (8,9). The greater activity of ARs with shorter Q tract lengths may be due to effects at several steps in receptor activation. A requisite intramolecular interaction between the AR N-terminal and ligand binding domains (N/C interaction) is affected by Q tract length and modulates protein function and stability (10,11). Q tract length also affects interaction with cofactors, including p160 coactivators such as AIB1 and SWI/SNF chromatin remodeling components (10,12). Intriguingly, the Ras-related nuclear protein (RAN)/ARA24 is sensitive to Q tract length, showing increased interaction and activation of ARs with shorter Q tracts, perhaps via effects on nuclear transit (13).

Although the Q tract has functional significance in vitro, and its extreme expansion causes SBMA, it is unclear if length variation within the normal range affects androgen-influenced traits, particularly pathologies such as prostate cancer. Evidence suggesting such a link includes greater prevalence of short Q tract alleles in the high-risk African-American population (14) and contraction of Q tracts in some prostate tumors compared with adjacent normal tissue (15,16). Numerous epidemiological studies have associated shorter Q tract length with increased prostate cancer risk, earlier onset or more aggressive disease (14,17,18). However, other studies have found no such association (19,20; reviewed in 21). These contrasting results may stem from small sample sizes, disparate methods of disease detection, genetic heterogeneity and confounding gene–gene and gene–environment interactions.

In order to investigate Q tract length effects experimentally, we developed a mouse model by converting murine $Ar$ to the human sequence through gene targeting (22). Three lines of humanized AR (h/mAR) mice were created with short (12Q), median (21Q) or long (48Q) Q tracts in the X-linked $Ar$ gene. All lines are physiologically normal with phenotypically similar androgen-influenced traits, but some small differences in AR target gene expression are detectable. However, when h/mAR mice were crossed with the TRAMP (transgenic adenocarcinoma of the mouse prostate) cancer model (23), significant differences in tumorigenesis between Q tract variants were evident at 29 weeks of age (22).

To examine in detail the AR Q tract effect on prostate cancer course, we compare here the development of prostatic intraepithelial neoplasia (PIN), time course of initial tumor detection and survival in h/mAR-TRAMP mice with different AR alleles. Q tract variation had a significant impact on cancer, with the stronger 12Q AR driving earlier oncogenesis but slower progression of more differentiated disease. In h/mAR-TRAMP mice that were castrated at 12 weeks of age, Q tract length remarkably also affected androgen-independent disease, but in directions opposite to those in intact mice. This suggests that differences in AR activity may direct distinct pathways of progression following androgen depletion. These h/mAR mice provide a unique paradigm for investigations of AR and the androgen axis in both hormone-dependent and -independent prostate cancer.

RESULTS

Generation of h/mAR-TRAMP mice and monitoring of tumorigenesis

H/mAR mice were created by replacing most of mouse $Ar$ exon 1 with human $Ar$ sequence (encoding amino acids 31–484) through homologous recombination in embryonic stem cells (22). This results in an AR nearly identical to hAR in coding regions but maintaining mouse chromosomal regulatory sequences (Fig. 1A). Three lines of h/mAR mice with alleles containing 12, 21 or 48 CAG repeats were created to represent short, median and long Q tract lengths as found in human populations. Besides the Q tract, h/mAR differs by 15% in N-terminal protein sequence from wild-type mAR, but gross physiology and reproduction of these mice is indistinguishable from wild-type littermates, suggesting the humanized AR can functionally substitute for mAR (22). Since spontaneous prostate cancer is extremely rare in wild-type mice and has not been found in aged h/mAR mice, these strains were crossed with the TRAMP model to initiate disease. TRAMP mice carry a SV40 T antigen (Tag) transgene driven by the prostatic epithelium-specific probasin promoter (23). Males develop PIN by 12 weeks of age and die from prostate cancer in 4 months to 1 year (23,24).

Tumor development was monitored in h/mAR-TRAMP mice and control mAR/Tag+ littermates from 12 weeks of age by weekly abdominal palpation by two independent observers. Tumors were first detectable at about 1–1.5 cm in diameter by increased tissue density just caudal to the bladder and were scored Status 1. Tumors measuring 1.5–2.5 cm and greater than 2.5 cm were scored as Status 2 and Status 3, respectively (Fig. 1B, C). To confirm palpation accuracy, a subset of 28 animals was monitored concurrently by magnetic resonance imaging (MRI) every 2 weeks for 3 months. Tumors were initially detected by MRI when prostate volume, normally $<30 \text{ mm}^3$, sharply increased to 100 mm$^3$ or greater, similar to previous MRI analysis of TRAMP tumorigenesis (25). During monitoring of this subset, eight animals developed tumors that were detected by both palpation and MRI, with a median of 3 days later for palpation (Fig. 1B; minimum of 5 days earlier and maximum of 16 days later for detection by MRI versus palpation). Tumors were not detected by either method in the remaining mice. Because age of detection was similar with both methods, palpation was continued as the more time and cost efficient means of assessment.

Q tract length impacts development of PIN in h/mAR-TRAMP mice

PIN in mice is considered the precursor to prostate carcinoma (26). To test whether its development reflected any differential
risk conferred by AR Q tract alleles, prostates of 12-week-old h/mAR-TRAMP mice were examined (n = 10 per genotype). PIN lesions were characterized by epithelial tufting, amphophilic cytoplasm, nuclear hyperchromasia, nuclear stratification, mitoses, apoptotic debris and micropapillary and cribriform architecture (Fig. 2A) (24,26). Low grade PIN had minimal changes relative to normal prostate and was not recorded. High grade PIN (HGPIN) was separated into two categories, PIN2 and PIN3, the latter distinguished by expansion of the gland with cribriform proliferation and more pronounced hyperchromasia, mitosis and apoptosis. Each h/mAR-TRAMP mouse examined had some HGPIN at 12 weeks of age, but PIN3 was only detected in 12Q (2 of 10) and 21Q (3 of 10), and not in 48Q, mice. PIN lesions occurred in all prostatic lobes but were most easily recognized in the dorsal lobe.

The amount of available dorsal lobe associated with PIN was scored into categories 1–10 by estimating the approximate percentage PIN involvement, by a pathologist blinded to genotype (Fig. 2B). Mice varied in amount of PIN involvement per prostate but 12Q mice clustered more in the upper range (7 of 10 mice in category 6 or above, Fig. 2C), while the majority of 48Q mice clustered at a lower range (6 of 10 in category 4) (mean values of 6 for 12Q, 5.5 for 21Q, 4.9 for 48Q). AR Q tract length therefore subtly affected presence of PIN by 12 weeks, with shorter tracts promoting somewhat more and higher grade PIN than longer tracts. This trend of Q tract length to affect neoplasia might subsequently be amplified by downstream events impacting actual tumor growth. The variation in PIN between individuals of the same genotype implies that additional stochastic factors also influence oncogenesis.

In immunohistochemistry (IHC) of sequential prostate sections from the 12-week-old mice, AR was detected in stromal and epithelial nuclei similarly in all genotypes (Fig. 2D). The somewhat more intense appearance in areas of PIN was most likely due to higher cell density. In contrast, Tag was prominent in areas involved by PIN but less evident in normal epithelium, underscoring association of Tag with...
neoplastic transformation in TRAMP. Subtle quantitative differences in Tag expression that might underlie variation in PIN levels are not detectable by IHC but may exist and may stem from AR allelic strength. Previous Q-PCR analysis of prostate RNA from the parental strains shows expression of probasin, whose promoter drives the Tag transgene, varies with AR genotype, while AR levels in these strains are equivalent (22).

**Q tract length influences prostate cancer progression in intact h/mAR-TRAMP mice**

Tumorigenesis and survival were monitored in hormonally intact TRAMP mice bearing h/mAR or wild-type mAR alleles. The Kaplan–Meier analysis revealed a significant difference between genotypes in time to tumor detection by palpation (P-value<0.0001; Fig. 3, upper panel). Previously we reported that at 29 weeks of age ~85% of 12Q, 50% of 21Q and 30% of 48Q mice had a palpable tumor or had already died (22). The complete analysis here showed that tumors were initially palpable over a similar age range in mAR, 12Q and 21Q mice, but the median age of detection was more than 10 weeks later for 48Q mice (Table 1). 12Q and 21Q mice were close in median age of tumor detection (25 versus 27 weeks, respectively) but by 29 weeks most 12Q mice had palpable tumors compared with only half of the 21Q mice.

Similar to tumor detection, age of death in 48Q mice was also significantly delayed as determined by Kaplan–Meier analysis (P-value<0.0001) (Fig. 3, middle panel), with a median 13 weeks later than 12Q and at least 23 weeks later than 21Q and mAR mice. Despite a similar age of tumor detection for mAR, 21Q and 12Q animals, 12Q mice had a median survival much longer than mAR and 21Q. The combination of slightly greater PIN levels at 12 weeks, presence of more tumors at 29 weeks and overall longer survival suggests that tumors might initiate earlier in 12Q mice but grow more slowly. Therefore, differences in age of death in h/mAR Q tract variants were due to differences in tumor progression as well as initiation. A measure of the rate of tumor progression is the length of time with disease (Fig. 3, lower panel), defined here as the length of time between initial tumor detection by palpation and death. This time with disease was more variable but significantly longer overall in 12Q and 48Q animals compared with 21Q and mAR (P-value<0.05) (Fig. 3, lower panel).

**Hallmarks of tumor progression in intact h/mAR-TRAMP mice**

Despite relative genetic homogeneity of h/mAR-TRAMP mice, the age of tumor detection and time with disease were variable within each genotype. To investigate the role of AR in this variation, a tissue microarray was constructed with three cores from each of 10 end-stage tumors per genotype. Tumors from 12Q and 48Q mice with a long disease length were moderately or well differentiated, with high levels of AR in the epithelium and stroma revealed by IHC (Fig. 4A). In contrast, tumors with shorter disease lengths were poorly differentiated or undifferentiated, with very little AR detected in 12Q tumors and heterogeneous AR staining in 48Q tumors. This immunoreactivity was nuclear and occurred in fewer cells from tumors in mice with shorter compared with longer disease length. Tumors from 21Q and mAR groups, with overall shorter time with disease, were mostly poorly differentiated with heterogeneous AR staining. Therefore, high levels of AR were associated with greater differentiation and slower progression in all h/mAR-TRAMP tumors, and overall this
Table 1. Prostate cancer progression in intact h/mAR-TRAMP mice

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Median age tumor detection (weeks)b</th>
<th>Median age death (weeks)c</th>
<th>Median length of disease (days)d</th>
</tr>
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<tr>
<td>Early euthanasiaa</td>
<td>Moribund</td>
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<tr>
<td>12Q</td>
<td>0</td>
<td>13</td>
<td>25 (17, 47)</td>
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<tr>
<td>48Q</td>
<td>6</td>
<td>13</td>
<td>37 (24, 49)</td>
</tr>
<tr>
<td>mAR</td>
<td>13</td>
<td>13</td>
<td>26 (16, 35)</td>
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One 12Q, two 21Q and two 48Q mice died from metastatic disease without a palpable tumor. Median value (minimum value, maximum value).

aEarly euthanasia includes mice that died of causes other than prostate cancer, including 21Q and mAR mice (10 each) that were sacrificed for a 29-week time point.
bIncludes only mice that developed a palpable tumor (euthanized at 29 weeks and moribund).
cIncludes only those that died of prostate cancer.
dIncludes mice that both developed a palpable tumor and died from disease.

Figure 4. Expression of AR and markers of prostate cancer progression in intact h/mAR-TRAMP mice. (A) End-stage tumors from moribund mice (n = 10 per genotype) were used to create a tissue microarray that was stained for AR. Each panel is a representative section with the age of initial tumor detection and the age of death (in weeks) listed in the upper right black box, in order of increasing disease length. AR expression was low in rapidly progressing tumors (short disease length) of all genotypes. 12Q and 48Q tumors of long disease length (slow progression) had greater differentiation and higher AR expression. 21Q and mAR groups had less variation in disease length so less differential in appearance and AR expression. Bar = 100 μm. (B) Synaptophysin (Syn), clusterin (Clu) and SV40 T antigen (Tag) are shown in samples on the tissue microarray, with vertical columns of panels from the same tumor; age of initial tumor detection and age of death (in weeks) are indicated at the upper right. A representative tumor from each group with short or long disease length (D.L.) is shown. Bar = 100 μm.
was influenced by Q tract length. This was corroborated by analysis of AR levels by Western blot analysis (see Supplementary Material, Fig. S1) and Q-PCR (see in what follows). Tag presence was heterogeneous regardless of AR levels or disease length (Fig. 4B), corroborating uncoupling from AR regulation in advanced tumors (24).

In man, neuroendocrine differentiation is associated with aggressive, androgen-independent prostate cancer (27). Although the origin of these cells is unclear, they are not thought to express AR (28). Neuroendocrine cells are also associated with aggressive disease in TRAMP where they can express AR (24). To investigate whether neuroendocrine cells represent a phenotypic switch in progression, we assayed expression of synaptophysin, a neuroendocrine marker, on the tissue microarray. Most 21Q tumors (9 of 10) showed synaptophysin staining (Fig. 4B), with concurrent AR staining in six tumors and one positive for AR but not synaptophysin. 48Q mice showed the least synaptophysin staining (4 of 10 tumors, two of which had concurrent AR staining); five 48Q tumors were positive for AR but not synaptophysin while one expressed neither marker. Interestingly, tumors from 12Q mice were more similar to a human phenotype in that AR and synaptophysin expression did not coincide—5 of 10 tumors were positive for synaptophysin but negative for AR, while the remaining five tumors lacked synaptophysin but expressed AR. Overall, synaptophysin expression in these tumors appeared to be associated with poor differentiation and rapid progression.

Expression of clusterin was examined since this glycoprotein is sometimes upregulated in androgen-independent prostate cancer and may be anti-apoptotic (29). In well-differentiated h/mAR-TRAMP tumors, clusterin was present in the cytoplasm of epithelial cells (Fig. 4B, 12Q and 48Q tumors with long length of disease). Undifferentiated tumors showed little clusterin expression (Fig. 4B, short disease length). As previously observed in TRAMP mice (30), this suggests that clusterin expression is associated with more differentiated tumors.

Q tract length inversely impacts androgen-independent disease

To probe whether the AR Q tract influenced androgen-independent cancer, mice were castrated at 12 weeks when PIN lesions are already present. Tumors still arise from these pre-existing lesions despite lack of androgen, and thus bear similarity to those in men following hormone ablation. One might expect Q tract length to have little influence in these tumors since hormone would only drive AR prior to castration. Remarkably, Q tract length affected the age of tumor detection in castrated mice, and in a direction opposite to that in intact mice. Kaplan–Meier analysis revealed a trend (P-value = 0.079) towards delayed tumor detection for 12Q mice compared with 21Q and 48Q groups (Fig. 5, upper panel). 21Q and 48Q mice did not differ significantly in time to palpation. This corroborated assessment at 29 weeks of age, when only ~15% of 12Q mice had a palpable tumor, whereas ~40% of 21Q and 50% of 48Q mice had tumors or had already died. Age of death, although not significantly different (P-value = 0.511), showed a trend similar to age of tumor detection (Fig. 5, lower panel). Following castration, survival of 48Q mice was most similar to 21Q mice, whereas mAR, instead of being indistinguishable from the median human allele as in intact mice, exhibited a disease course intermediate between 12Q and the other mice (see Table 2). This did not reach statistical significance, but could reflect species differences in the AR N-terminus other than the Q tract.
Length of time with disease, assayable for those with a palpable tumor, was less variable and shorter in all castrate compared with intact groups, and was longer after initial tumor detection for 12Q mice (median 40 days) compared with 21Q (median 22 days) or 48Q (median 29 days) mice (Fig. 5, lower panel). Several animals either never developed palpable tumors (and so were not included in analysis of disease length) or had palpable but small tumors and became moribund due to heavy metastatic burden, identified on necropsy. The proportion of these cases was especially notable for castrated 12Q mice, where about half died of metastatic disease (6 of 13), compared with 1 of 11 21Q and 1 of 8 48Q castrated mice ($P = 0.06$ by Fisher’s Exact Test). 12Q mice that succumbed to metastasis died at similar ages as those with large primary tumors.

### Tumor differentiation and AR expression in castrated h/mAR-TRAMP mice

AR in androgen-independent disease was examined on the tissue microarray for 7, 8 and 6 tumors, respectively, from 12Q, 21Q and 48Q castrated mice. All of these end-stage tumors were detected by palpation except for three from 12Q mice that died of metastasis with only small primary tumors. Castrated mice generally had a short survival time once a tumor was palpable, and most tumors were undifferentiated with low or heterogeneous AR expression (Fig. 6A). Exceptions were the prostates from the castrated 12Q mice that died due to metastasis, which had areas of well-to-moderately differentiated carcinoma with high AR expression. In most castrated mice, the heterogeneous AR staining was frequently nuclear despite the lack of testosterone (Fig. 6B). However, the small tumors of 12Q mice dying of metastasis had evident cytoplasmic as well as nuclear staining, typical of AR in the absence of androgens.

Clusterin expression was also examined in castrated h/mAR-TRAMP mice since its upregulation has been noted after androgen ablation in man (29). Similar to intact mice, undifferentiated tumors expressed only low levels of clusterin regardless of AR allele, whereas the more differentiated 12Q tumors showed epithelial clusterin expression (Fig. 6C). Thus clusterin may not be a factor in androgen-independent progression in h/mAR-TRAMP mice.

### AR mRNA levels vary with disease length in intact but not castrated mice

AR was assessed by real-time RT–PCR to obtain a more quantitative view than provided by IHC. In intact mice, tumor AR mRNA levels varied widely within each genotype but, in accord with IHC, mice with longer length of disease tended to have higher AR expression, 2–4-fold greater than normal prostate (Fig. 7). Castrated mice had AR levels that were generally less than normal prostate. These results were corroborated by Western blot analysis (see Supplementary Material, Fig. S1). Thus regardless of allele or hormonal status, greater AR mRNA levels corresponded to slower progression of disease while low AR mRNA levels coincided with rapid progression. While difficult to infer specific mechanisms from these end-stage tumors, AR in general, by type and amount, influenced disease course.

### Effect of castration within genotypes reveals differential response to therapy

Comparison of intact and castrated mice of the same genotype highlights the impact of Q tract length (Fig. 8). In 12Q mice, not AR, while two small well-differentiated tumors expressed AR but not synaptophysin. One small moderately differentiated tumor showed both synaptophysin and AR expression. Castrated 21Q mice also had slightly more synaptophysin expression than intact animals (6 positive tumors of 8). Four had concurrent AR expression while two that were positive for synaptophysin lacked AR. In contrast, none of the 6 tumors from castrated 48Q mice expressed synaptophysin. Although castrated 21Q and 48Q mice had similar ages of tumor detection and death, and similar tumor histology, they differed substantially in synaptophysin expression. These varied expression patterns may reflect alternative pathways of tumor progression, directed in these mice by differential AR actions impacted by Q tract length.

Footnotes a–d are the same as for Table 1. Six 12Q, one 21Q, one 48Q and six mAR mice died of metastatic disease without a palpable tumor. Median value (minimum value, maximum value).
tumor detection was significantly delayed by castration ($P$-value < 0.0001). However, there was no effect of castration on survival (not shown, $P$-value = .386; see Tables 1 and 2), consistent with the long disease length for intact 12Q mice. While castration did not result in a significant difference overall in tumor detection for 21Q mice, some presented with earlier palpable tumors relative to intact 21Q, similar to previous findings in TRAMP mice (32). Most surprisingly, while 48Q mice did not show a difference in time to tumor detection, castration resulted in more rapid tumor progression and significantly shorter survival time than intact 48Q mice ($P = 0.011$, not shown; see Tables 1 and 2). Although the effect of Q tract length on treatment response is not well studied in man, a short Q tract length was associated with positive response to early androgen ablation in mice.
DISCUSSION

The polymorphic Q tract of AR was first implicated as a risk factor in prostate cancer because its length correlates inversely with significant differences in disease incidence between racial groups (14). However, epidemiological studies have reached discordant conclusions. We examined the effect of Q tract length in an experimental system genetically less complex than the human population—an allelic series of ‘humanized’ AR mice carrying a transgenic oncogene. In intact h/mAR-TRAMP mice, our results support the hypothesis that tract length correlates inversely with cancer risk—the AR allele with the long 48Q tract is significantly associated with delayed disease detection while the short 12Q tract allele is associated with earlier neoplasia. Q tract length also influences progression, although not in a linear fashion, since both 12Q and 48Q mice survive longer than 21Q (or mAR) mice. Remarkably, Q tract length also appears to affect disease course in castrated mice that model androgen-independent progression. In this case, the short Q tract confers later tumor detection and death, and unlike other groups, more than half of the deaths are from metastatic disease. This allelic series presents the opportunity to discern mechanisms underlying Q tract influence in androgen-dependent versus androgen-independent disease and to dissect the means by which different ARs drive apparently distinct oncogenic pathways.

Epidemiological data indicate that Q tract length effects vary with the population studied, evidencing genetic heterogeneity and environmental factors (33). Conflicting results also likely stem from varied definition of ‘short’ versus ‘long’ alleles, whereas in actuality extremes within the normal range may account for most differences (10,34). Further, the method of disease detection may introduce bias. Studies that do not rely on PSA screening show stronger association between Q tract length and cancer risk, suggesting a link to aggressive but not indolent disease (35). The h/mAR mice avoid these difficulties since they have a relatively homogeneous genetic background, controlled environment, and show no prostate pathology in the absence of Tag expression (22). Therefore differences detected in this model are more likely to be due to Q tract polymorphism, and may even be accentuated by the uniformity of other parameters.

In man, allelic variation in multiple androgen axis genes, particularly Ar, cytochrome P450 (CYP17) and steroid-5-α-reductase type 2, impact prostate carcinogenesis

Figure 7. AR mRNA expression is associated with length of disease in intact but not castrated h/mAR-TRAMP mice. AR tumor mRNA was assessed by real-time RT–PCR. Each bar represents expression in a single tumor relative to normal prostate, which was RNA pooled from several 21Q h/mAR prostates (set at fold difference = 1). Samples were ordered by increasing disease length (D.L.—from tumor detection to death) within each group. * indicates an animal that died due to metastasis. Values are given as mean ± standard deviation.

Figure 8. Q tract length impacts differential response to therapy. Proportion of intact (gray) and castrated (black) h/mAR-TRAMP mice without a palpable tumor is compared by genotype, using data of Figures 3 and 5. Castrated 12Q mice develop palpable tumors significantly later (P-value = 0.0001) than intact animals. Some 21Q mice benefit from androgen ablation with a delay in time to tumor (P = 0.325), whereas 48Q mice show almost no difference between intact and castrate groups (P = 0.940).
additively, emphasizing the multigenic nature of this cancer (36,37). In the h/mAR mice, varying Q tract length may create differential strengths of the androgen axis. This is exemplified by effects in intact mice, where 12Q h/mAR-TRAMP have slightly more PIN at 12 weeks, while 48Q mice have delayed tumor onset and death. The mechanism underlying these effects is likely the impact of Q tract length on transcription of AR-dependent genes. In transcription, shorter Q tract length produces increased AR levels (11), stronger N/C interaction (12) and greater coactivator recruitment (10), summing to greater AR transactivation. Q tract length effects on gene regulation are also detectable in vivo (22). Because the androgen-responsive probasin promoter drives Tag expression in TRAMP, a short Q tract AR may induce sufficient Tag to transform cells earlier than other alleles. This differential may be compounded by stromal AR, which controls paracrine factors that influence epithelial cell survival (38). Effects of AR in stroma as well as epithelia may enter into higher Tag expression in regions of PIN compared with normal epithelia. Although human cancer is not initiated by Tag, Q tract length may affect expression in man of AR target genes that are involved in early oncogenic events. An example of such a target is the recently identified, and commonly occurring, TMPRSS2:ETS fusion gene (39).

An intriguing finding is the effect of the Q tract following castration, which appears to be in a direction opposite to that in intact mice. This effect is most notable in the trend to delayed tumor detection for 12Q mice. If androgen ablation simply inactivated AR, subsequent progression might follow the trend established for PIN by 12 weeks (12Q>21Q>48Q) or reach equivalence between groups regardless of genotype. Reversal of the trend suggests that AR is still acting, although differently, in the castrate environment. As shown in vitro, the short tract AR may be hypersensitive to low androgen (12), coming from residual circulating hormone or intraprostatic synthesis. Q tract length may also impact AR phosphorylation, leading to ligand-independent activation, as can occur via pathways including MAPK and Akt (5). Regardless of the mechanism, AR signaling clearly remains an important determinant of progression even in androgen-independent cancer; in fact, under low hormone conditions the androgen axis may be even more critical (40). An intriguing inference is that mice with the more active AR fare better, at least in the short term, in opposing the strong Tag oncogene than those with weaker ARs.

Q tract length effects in androgen-independent disease are highlighted by comparing intact and castrated mice within genotypes, revealing a genetic component in differential response to therapy (Fig. 8). Whether Q tract length influences response to androgen ablation in humans is uncertain since a similar number of studies show poorer response with short (41–43) as with long Q tracts (44–46). Response is difficult to compare in humans due to heterogeneity of treatments, while these mice underwent androgen ablation at the same age and similar stage of disease, although earlier than treatment in most men. Early treatment may in fact drive selection for more aggressive tumors. This is supported by data suggesting androgen-independent cells exist prior to castration in TRAMP mice (32), and by results of the finasteride prevention trial in man, in which fewer tumors were found but more were of higher grade (47). Castrated 12Q mice develop disease later and survive longer than their intact counterparts, whereas castrated 48Q mice have shorter mean survival compared with intact animals. This difference suggests that AR strength may impact response to androgen ablation, with a stronger AR having more favorable outcome. In man, low testosterone prior to treatment, indicating a weak androgen axis as in 48Q mice, correlates with poor prognosis (48). Perhaps in some cases total androgen ablation may be worse than delayed or intermittent hormonal therapy, and may select for more aggressive tumors.

The influence of Q tract length on AR action is complex, reflecting the fact that AR itself has both stimulatory and inhibitory roles that vary with the cell and target gene context. Moreover, a simple linear relationship between Q tract length and AR activity may not exist, and different alleles may be optimal in different situations for different reasons. As an example, well-differentiated slow-growing tumors occur in intact 12Q and 48Q, but not 21Q, mice. In prostate development, an initial proliferative capacity of AR transitions to one that is largely differentiative; in oncogenesis, the balance of these opposing functions tips in the opposite direction. During cancer ontogeny, AR’s predominant role may shift from tumor suppressor to oncogene (49). AR signaling in the normal prostate maintains a low proliferation rate that may be enhanced by a short tract allele to spur initial tumor growth. However, a more active AR may also more effectively maintain differentiation, perhaps particularly when ligand is low, as suggested by tumors of castrated 12Q mice. The function of AR may also vary in stromal versus epithelial compartments. Upon androgen stimulation, prostate stromal cells produce paracrine growth factors that drive epithelial proliferation (50). In contrast, epithelial cells differentiate with hormone and proliferation is reduced (51). Q tract variation may differentially affect AR activity in these two cell types, likely due to differences in amounts or types of cofactors and array of signaling pathways in these cells. Given this complexity, the h/mAR variants provide a unique paradigm in which to investigate androgen-dependent and -independent AR functions in prostate cancer, to elucidate mechanisms underlying their differential response to hormonal therapy and for pre-clinical testing of novel therapeutics targeted at the human AR.

MATERIALS AND METHODS

Mouse breeding and care

Heterozygous h/mAR female mice on a mixed B6;129/Sv background (generation F4) were crossed to homozygous TRAMP mice on the C57BL/6J background (gift of M. Sanda). Male progeny were genotyped as previously described (22) and randomly assigned to intact or castrated groups. The latter were orchiectomized at 12 weeks of age, performed under isoflurane anesthesia through a midline scrotal incision. MRI imaging was performed by the Michigan Small Animal Imaging Resource at the University of Michigan. Mice were monitored daily for morbidity and mortality and were euthanized when moribund, as determined by immobility, signs of urinary blockage, prolonged hunched posture, cachexia or dyspnea. 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.

Tissue preparation and histology

At necropsy, the genitourinary tract (bladder, seminal vesicles and prostate) was removed en bloc and weighed. Sections of primary prostate tumor and grossly visible metastases were fixed in 10% (vol/vol) buffered formalin for 24 h, transferred to 70% (vol/vol) ethanol, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin using standard methods. Additional portions of harvested tissues were preserved in RNAlater buffer (Ambion Inc., Austin, TX) or frozen in liquid nitrogen.

For 12 week mice, the genitourinary tract was removed en bloc and fixed in formalin for 24 h. A transverse incision was made through the urethra at the level of the prostate and both portions were paraffin embedded as described (26). Two levels of hematoxylin and eosin stained slides from each animal were reviewed by the pathologist (K.W.) who was blinded to genotype. The percent of dorsal lobe involved in PIN was visually estimated with a 1–10 scale of −10% intervals. Data was recorded for first level and confirmed by review of second level.

Real-time RT–PCR analysis

Total RNA was isolated using RNeasy columns (Qiagen Inc., Valencia, CA) and 1 μg was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Real-time PCR assays used SYBR Green PCR master mix (Applied Biosystems) and 10 ng cDNA. AR primers were designed with Primer Express software and used at 900 nM each (forward primer: 5’-GCGGTCCCTCATAATGTCAACT-3’, reverse primer: 5’-GAGACTTGTGACATCGGTACTC-3’). GAPDH primer sequences were from PrimerBank (ID 6679937a1) and were used as described (52). Each sample was measured in duplicate with two independent assays, values were normalized to GAPDH, and relative expression was calculated by the standard curve method.

Immunohistochemistry

IHC was performed as reported previously (53). Briefly, paraffin-embedded sections were deparaffinized, rehydrated and boiled in 0.01 M citrate buffer (pH 6.0) for 30 min. Sections were incubated overnight at 4°C with antibodies against AR (N20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:500 dilution, SV40 Tag (pAb416, Calbiochem, San Diego, CA) at 1:100 dilution, synaptophysin (18-0130, Zymed Labs Inc., South San Francisco, CA) at 1:200 dilution or clusterin (M18, Santa Cruz Biotechnology, Inc.) at 1:600 dilution. Sections were then incubated with species-specific biotinylated secondary antibody and stained with an avidin–biotin peroxidase system (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) using diaminobenzidine tetra-chloride as a chromogen.

Statistical analysis

We used the Kaplan–Meier method to calculate the proportion of mice without a palpable primary tumor. The endpoint of interest was the presence of a palpable tumor. We considered mice that (prior to palpable manifestation of prostate cancer) succumbed to disease or were sacrificed censored at their time of death. We also used the Kaplan–Meier method to calculate prostate cancer-free survival rates. The endpoint of interest was death due to prostate cancer (from primary tumor or metastatic spread). Mice that succumbed due to a competing cause of death or were sacrificed prior to palpable manifestation of prostate cancer were considered censored at their time of death, with one exception. Castrated mice with palpable tumors that were sacrificed (due to morbidity) but were subsequently discovered to have a 2+ size primary tumor with metastases or a 3+ size primary tumor (with or without metastases) were considered as events (and not censored) at the time they were sacrificed.

Using log-rank statistics, we compared the proportion of mice without a palpable primary tumor and prostate cancer-free survival rates (1) between AR genotype classes (mAR, 12Q h/mAR, 21Q h/mAR and 48Q h/mAR) within intact or castrated mice and (2) between intact and castrated mice within each AR genotype class. All analyses were conducted using the R language (version 2.1.1). P-values <0.05 were considered statistically significant. Due to the small number of mice in some groups, we also tested a permutation procedure to assess the significance of each log-rank statistic. For each statistic, we created 10 000 permuted data sets by randomly permuting the genotype (or hormonal) class labels of the mice, while leaving intact their tumor and survival data. We then calculated the log-rank statistic for each permuted data set. For each statistic, the P-value was calculated as the proportion of permuted test statistics that were as large as or larger than the observed value. Permutated P-values did not differ substantially from log-rank or asymptotic P-values.

Other statistical analyses were performed using SAS software version 9.1 (SAS Institute, Cary, NC).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.
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