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13. ABSTRACT (Maximum 200 Words)

Androgen receptor (AR) is a critical component in prostate oncogenesis. AR alleles varying in length of the Nterminal glutamine (Q) tract carry distinct disease risks. Mutation of AR in tumors may influence androgen independence and resistance to treatment. Difficulties studying cancer initiation and progression are partly overcome with transgenic mouse tumor models. However, the mouse AR N-terminus differs significantly from human. To evaluate the role of the glutamine tract, and to identify relevant sites in the N-terminus whose mutation can lead to androgen independence, we have "humanized" the mouse by converting its AR gene to the human sequence (h/mAR). We have done this using homologous recombination in embryonic stem cells and have established mouse lines with AR alleles with 12, 21, or 41 Qs. Mice bearing these alleles are normal in fertility, gross physiology and most molecular analyses. However, expression of some specific androgen target genes suggests subtle distinctions exist. Differences are more apparent when placed on the TRAMP background that provides a transgene for prostate oncogenesis. Disease progression in castrated mice (mimicking androgenindependence) is more aggressive with humanized than wild type mouse AR alleles. The basis of this is being assessed histologically by tissue microarray and at the molecular level by sequencing AR cDNAs for mutations.

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INTRODUCTION

Development and progression of prostate cancer (PCa) depend on genetic and environmental factors that are still poorly defined. One thing all tumors share, however, is an initial dependence on androgen for growth [1]. Polymorphisms in androgen receptor (AR) may impact risk of disease, and somatic mutations may affect progression and response to therapy [2-4]. In most cases, hormonal therapy is initially successful, but tumors ultimately become androgen-independent and resistant to treatment. Despite androgen independence, AR levels in the tumor remain high and the AR signaling pathway is intact, revealing a continued role of AR in the disease process. AR molecular genetics may be informative for two crucial problems in PCa: **1) How do polymorphisms in AR lead to greater risk of disease? 2) How do somatic mutations in AR during tumor growth circumvent hormone ablation?** This project addresses these questions for the human receptor in a transgenic mouse prostate cancer model, allowing initiation, progression and treatment of disease to be integrated experimentally. The mechanisms by which genetic variation alters AR action may reveal new molecular markers in treatment, and, ultimately, novel targets for therapy.

BODY

Our hypothesis is that **genetic variation in human AR affects the initiation and progression of prostate cancer.** Germline variation may affect initiation of disease, while somatic mutations may drive androgen-independent growth. To confirm this hypothesis, our first major objective was to replace the mouse AR with human alleles to study their effects in general mouse physiology and in a transgenic tumor model (Transgenic Adenocarcinoma of the Mouse Prostate, or TRAMP [5]). Since Q (glutamine) tract length enters into PCa risk, we are comparing varying length Q tract hAR alleles. We also are comparing mutations arising in androgen-dependent vs. –independent disease, to identify sites correlating with AR function. Our aims underlying the Statement of Work remain as follows:

Aim I. To study the role in PCa of polymorphisms in human *Ar*, m*Ar* will be "humanized" by homologous recombination in embryonic stem cells, to create three *h/mAr* alleles differing in glutamine tract length (12Q, 21Q, 41Q). Differences in androgen action (fertility, behavior, molecular markers) and spontaneous prostate cancer will be studied in mice with *h/mAr* alleles.

Aim II. To determine the role of human AR variants on PCa initiation, *h/mAr* alleles will be placed on the TRAMP background for transgene-induced oncogenesis. Effect of the Q tract will be assessed on prostate pathophysiology and gene expression by cDNA microarray.

Aim III. To determine the role of AR variation on PCa progression, spontaneous mutations will be identified in AR cDNAs from castrated (androgen-independent) vs. intact *h/mAr*-TRAMP mice. The effect of mutations will be determined by introduction into ARs for transfection analysis, with and without coactivators. The effect of mutations on the oncogenic potential of prostate cells will be tested by tumor formation and metastasis in SCID mice.

Each Aim corresponds to a Task within the Statement of Work. Aim I is essentially completed and we are analyzing data for preparation of a manuscript. The 12Q h/mAR strain lagged in construction but has now caught up and we are beginning to get tumor data from those mice crossed to TRAMP, to add to the complete data sets for the 21Q and 41Q strains. Aim III is underway, with some cDNA sequencing already accomplished. Some of the AR mutations we have found have been noted previously; novel mutations will be functionally tested as planned. Thus this project should be completed in line with the original Aims by the end of the (extended) project period. Significant findings are summarized below, with figures included in the appendix.

All three mouse lines were derived in the 129/Sv background, the strain commonly used to produce ES cells for homologous recombination. They have been backcrossed at least 5 generations onto the strain more commonly used for experimentation, C57BL/6J, to eliminate strain effects. Animals from this 5th generation have been set aside for life-span studies and to assess cohorts at 6 month intervals. For all 3 h/mAR strains, we have noted no differences from wild type littermates in gross appearance, behavior, or fertility. Hormone assays for serum levels of testosterone and estrogen have also not revealed significant differences. However, hormone levels are subject to wide variation, so we are currently measuring seminal vesicle weight as a better indicator of testosterone action. Since we were not initially measuring this parameter, sufficient mice to determine significance are being aged to 6 months.

cDNA microarray analysis was used to assess global differences in gene expression in androgen responsive tissues of the humanized 21Q mice compared to wild type littermates. No significant or reproducible differences have been found with this methodology for a mouse cDNA array prepared and analyzed in the Microarray Core of the University of Michigan Cancer Center. An example of the comparative data is shown in Fig. 1, Appendix. In contrast to this global screen, some individual genes that are known to be androgen responsive in preliminary analysis have shown differences by Northern blot, and we are currently doing more definitive analysis with more individuals. At least for cyp2d9 and Mups in liver, levels appear somewhat higher in the 21Q h/mAR mice, which would correlate with the human allele being a more potent transcription factor *in vivo*, as it is *in vitro* (unpublished data).

A possible variation in these strains is level of expression of AR itself. Since the 5′ flanking and intronic sequences of the AR gene are identical in all strains, differences in expression level would probably be due to posttranscriptional effects, for instance mRNA or protein stability. We are assessing mRNA levels by Q-RT-PCR (Taqman) and protein levels by Western blotting. Fig. 2 (Appendix) is a Western of AR levels in kidney and testis for several individual wild type and 21Q h/mAR and 41Q h/mAR mice. AR protein levels indeed appear higher for the humanized alleles. This is being tallied for at least 6 individuals of each strain, in prostate and liver as well as testis and kidney, for mice at 6 months of age. Older animals will be tested as they attain specific time points. Both mRNA and protein stability in cell culture experiments have been shown to vary with AR glutamine tract length [8]. These subtle differences in AR levels may become significant in situations such as hormone-dependent disease (e.g., prostate cancer, see below), but are not sufficient to imbalance normal homeostasis or fertility.

These mice have been crossed onto the TRAMP background, to initiate cancer via prostate epithelial-driven T antigen expression. At the age of twelve weeks one cohort (12-17 mice) was castrated and another left intact. Tumor initiation and progression has been followed by external abdominal palpation, and more recently by magnetic resonance imaging (MRI). The MRI analysis allows us to detect tumors about two weeks earlier than manual palpation, but otherwise agrees with qualitative assessments about rate of tumor growth from palpation. Mice are sacrificed when they become moribund, and tumors collected for histological and molecular analysis. Tumors from the 12 Q allele mice are in the process of being collected, but the tumors of the 21 Q, 41 Q and wild type mice have all been harvested. While molecular analysis is ongoing, there are some intriguing results with respect to tumor progression, described below.

As noted by others working in the TRAMP model, castration (or treatment with flutamide) may delay but does not prevent prostate cancer, for about 70% of the treated (wild type) mice [6]. However, for about 30% of the mice, more aggressive disease occurs in the face of androgen ablation. This finding bears remarkable similarity to results of the SWOG trial in which men were treated with finasteride [7]. Tumor progression is thus heterogeneous in TRAMP mice as it is in men, despite the genetic homogeneity of a mouse model. When TRAMP mice bearing the 21 Q h/mAR allele were compared to littermates with wild type mAR, we found no remarkable differences in disease progression in intact mice (see Figs 3 and 4, Appendix). However, in the castrated group, the humanized mice showed a significant increase in early aggressive disease. Interestingly, intact 41Q h/mAR X TRAMP mice show resistance to disease relative to wild type and 21Q h/mAR mice, but show similarly aggressive disease as 21Q h/mAR mice following castration. One interpretation is that in intact mice the glutamine tract exerts an effect on initiation of disease, with a longer tract being protective, in agreement with human epidemiology and in vitro studies. However, in castrated mice (androgen-independent disease), sequences within the human AR N-terminus other than the Q-tract underlie the greater aggressivity compared to mAR.

Clearly data from the 12Q mice will prove very informative as it becomes available. We are currently comparing levels of AR in these samples by immunohistochemistry (Fig. 5, Appendix), and correlating this with the level of T-antigen expression. This latter parameter is critical, since the oncogene is driven by the AR-dependent probasin promoter, and thus level of T-antigen expression may depend on the strength of the AR allele. To compare these crucial markers semi-quantitatively, and to examine many additional downstream events and signaling pathways, we have constructed tissue microarrays with these tumors. These arrays, generated by the U. of M. Microarray Core should prove generally of great value for mouse tumor studies.

Finally, we are beginning to analyze AR mutations arising in these tumors, by sequencing the equivalent of 10 full-length cDNAs per tumor. We have had some technical difficulties due to the highly GC-rich nature of the N-terminus, but are now getting consistent results. We are finding 1-3 mutant ARs per tumor, in accord with previous observations [9], and thus far they are a mix of previously noted as well as novel mutations. The numbers are not yet great enough to confirm that the sites of mutations (i.e., LBD vs. N-terminus) are dependent on hormonal status (i.e., intact vs. castrated). In the next year this Aim will be completed. Examples of some mutations found thus far are shown in Fig. 6, Appendix.

In sum and in accord with our original plan, the third year of this project has largely been spent in establishing the third mouse strain for analysis, characterizing the strains at a gross level, and generating and collecting tumors from the TRAMP cross. These mouse strains function as predicted, in being physiologically normal but having subtle phenotypes when hormonal effects are examined in greater detail. We are particularly excited by the apparently great increase in aggressive disease with the humanized allele, as we would predict for the stronger human than mouse AR. Thus we are encouraged that these mice will be valuable for assessing the role of androgen receptor in prostate cancer initiation and progression, may provide better subjects for preclinical testing, and may lead to new treatments for disease.

KEY RESEARCH ACCOMPLISHMENTS

12Q, 21Q, and 41Q h/mAR mouse strains are established and have been crossed for at least 5 generations, with no gross abnormalities noted. F5 individuals at 6 months have been compared for molecular and endocrine markers, and phenotypic characterization of these strains is being compiled for publication. 21 Q and 41 Q humanized AR mice were crossed with TRAMP to generate prostate tumors, and all tumors have been collected. The humanized mice show a more aggressive form of disease than wild type littermates when castrated, confirming that the human AR gene has a subtly distinct activity than the mouse. 12Q h/mAR X TRAMP tumors are developing and will allow completion of comparison of the effect of short, average, or long Q-tracts on prostate cancer in a mouse model.

REPORTABLE OUTCOMES

A manuscript is in preparation on creation of the "humanized" AR mouse and comparison of wild type mice to the three h/mAR Q-tract strains (12Q, 21Q, 41Q).

We have presented our results at local poster sessions and informal seminars, and also as part of departmental colloquia at:

UCLA, Dept of Human Genetics, 1/12/04
USC, Norris Cancer Center, 1/13/04
Case Western Reserve Univeristy, Dept of Pharmacology, 4/12/04
NIEHS, Reproductive Endocrinology, 5/7/04
University of Minnesota. Dept of Biochem and Molecular Biology, 9/29/04

Talks were presented at the following meetings; the unpublished abstract for the Prouts Neck meeting is included in the Appendix:

Symposium on Androgen Action in the Prostate, Keystone, Mar 4-6, 2004
10th Prouts Neck Meeting on Prostate Cancer, Nov 4-7, 2004

An abstract has been submitted to the Endocrine Society for the annual Conference in June; the abstract is included in the Appendix.

The 21Q h/mAR mice are proving a valuable resource and are also being used for a project within the University of Michigan SPORE in Prostate Cancer, to determine effects of antiandrogens (flutamide, bicalutamide) on incidence of mutations in the humanized compared to mouse AR.

The targeting vector used to create the 21Q h/mAR mice has been modified in collaboration with two junior investigators to create mouse models for other studies. With Andrew Lieberman, a mouse model of Spinal and Bulbar Muscular Atrophy (SBMA, Kennedy's disease) has been created bearing a CAG tract expanded to 113 Q. With Jorge Iniguez, SUMO modification sites that down-regulate AR function have been knocked out. These studies have already led to additional grants for the junior investigators, where I serve as mentor (on Lieberman's KO8) or collaborator (for pending Iniguez funding).

CONCLUSIONS

Mouse strains carrying human rather than mouse AR sequences have been constructed. This allows direct testing of the role of AR glutamine tract variation in initiation of prostate cancer, which may help clarify contradictory results from epidemiological studies. Further, tumors initiated by transgenes in these mice will allow tracking the role of AR, and AR mutants, in resistance to antiandrogen therapy and androgen independent growth. The site of mutations in human AR sequence may lead to downstream interacting proteins that will be novel and perhaps more effective targets in new treatment strategies.

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APPENDICES

1) Abstract from 10th Prouts Neck Meeting on Prostate Cancer, Nov 4-7, 2004

2) Abstract submitted to the Endocrine Society Meeting, June 4-7, 2005

3) Figure 1 – Comparison of testis gene expression in humanized vs. wild type mice.

4) Figure 2 – AR protein levels in testis and kidney of humanized vs. wild type mice.

5) Figure 3 – Survival curves for TRAMP mice with humanized vs. wild type AR alleles.

6) Figure 4 – Comparison of tumor lethality at 29 wks of age for AR variant mice.

7) Figure 5 – AR immunohistochemistry of tumors from AR variant mice.

8) Figure 6 – Initial results of AR mutation analysis from TRAMP tumors.

Abstract for the 10th Prouts Neck Meeting on Prostate Cancer Nov 4-7, 2004

Human Androgen Receptor Genetic Variation and Prostate Cancer in Mice.

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Despite diverse genetic and environmental influences in prostate cancer, tumors initially depend on androgen for growth, reflecting crucial roles of the hormone receptor (AR) in gene regulation. Because of its importance in tumor progression, AR is a key target in therapy. Genetic polymorphisms in AR have been associated with risk, and somatic mutations occur in late disease. The significance of these variations has been difficult to study, partly due to lack of early stage patient samples and lack of animal models that address underlying mechanisms.

To test the oncogenic role of AR polymorphisms, and the function of somatic mutations in disease progression, we have converted the mouse AR gene to the human sequence, by gene targeting in embryonic stem cells. The mouse and human ARs are nearly identical in DNA and ligand binding domains, but diverge by 15% in the N-terminal transactivation domain, including in the extent and position of a polymorphic glutamine (Q) tract. The length of this tract is inversely correlated with tumor risk in man and receptor transactivation ability *in vitro*. The effect of AR alleles differing in glutamine tract length (12Q, 21Q and 41Q) is being tested in the context of the transgenic mouse tumor model, TRAMP, developed by Norman Greenberg. This also allows us to examine mutations that arise in AR during progression, in order to confirm and extend Greenberg's observation that AR mutations in TRAMP tumors occur in distinct receptor domains dependent on hormonal status. An intriguing result thus far is that in intact mice the mAR and 21Q hAR alleles allow similar PCa progression, whereas the 41Q hAR is more resistant to tumor development, in accord with human epidemiology. Thus these mice may reveal the basis of Q tract differences in PCa initiation. In contrast, in castrated mice, both human alleles produce more aggressive disease than mAR, suggesting N-terminal sequences other than the Q tract may influence androgen-independent progression. These mouse models may accentuate androgen-dependent and –independent disease and may distinguish roles of AR that vary with disease stage and liganded state of receptor.

ABSTRACT SUBMITTED TO THE ENDOCRINE SOCIETY FOR THE ANNUAL MEETING, JUNE, '05

Replacing the Mouse Androgen Receptor with Human Alleles Varying in Glutamine (CAG) Tract Length

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Within the androgen receptor (AR), polymorphic variations in the length of an N-terminal glutamine (Q) tract (CAG repeat) are associated with disease states including prostate cancer, infertility and, for extreme lengths, spinobulbar muscular atrophy. To determine how AR's Q tract length may influence prostate cancer initiation or progression and whether acquired mutations lead to androgen independence, we have converted the mouse AR sequence to that of man.

While mammalian ARs are nearly identical in the DNA and ligand binding domains, they differ by about 15% in the N-terminal amino acid sequence. The mouse AR Q tract is shifted by about 100 amino acids carboxy-ward of the human position and is disrupted by several histidines, while a glycine tract before the DBD is greatly abbreviated. To "humanize" the mouse AR gene, we created a targeting vector in which the human AR N-terminus (amino acids 35-466) replaced the equivalent mouse region of exon 1, and was flanked by mouse 5' untranslated and first intron sequences derived from genomic 129/SV DNA. Homologous recombination in embryonic stem cells resulted in an AR transcription unit in which human coding sequences are controlled by mouse regulatory elements.

Three mouse lines were created with short, average, or long Q tracts (12, 21, and 41 Q). At a gross level, humanized AR mice display normal fertility, body weight, reproductive anatomy, and behavior, indicating that human AR can functionally replace the mouse gene. Testosterone levels and prostate histology are not significantly different between glutamine tract variants at six months of age. However, studies of AR levels and expression of AR target genes show subtle differences between the glutamine tract variants. Further, studies evaluating the effect of Q tract length on prostate tumor onset and progression in the transgenic TRAMP model amplify the differences between these variants and in comparison to wild type mouse AR. Therefore, variation in glutamine tract length within the "normal" range may not greatly impact steady-state physiology, but may play a role in androgen-dependent diseases such as prostate cancer. These humanized AR mice are useful for modeling the role of the AR N-terminus in disease and for developing new AR-targeted therapeutic agents.

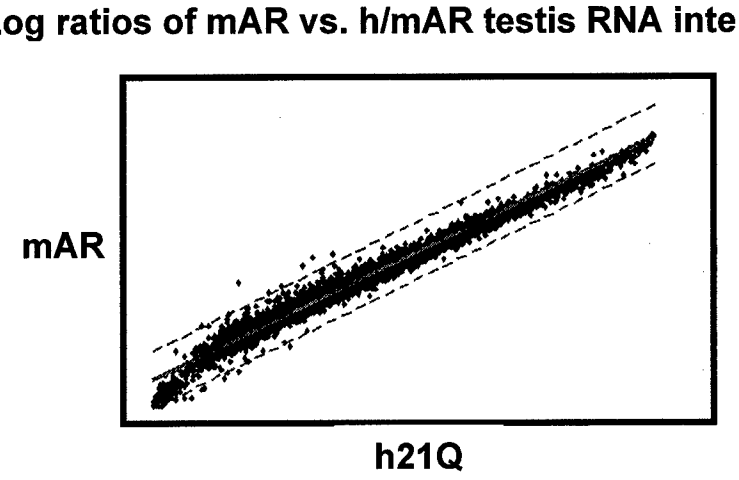

Figure 1 – Comparison of testis gene expression for 5000 mouse genes, for mice with the humanized 21Q allele (h21Q) versus their wild type littermates (3 mice per group). Log ratios of expression relative to the UniReference Standard fall mostly between the two outer lines, indicating less than two-fold variation, which is taken as not significant.

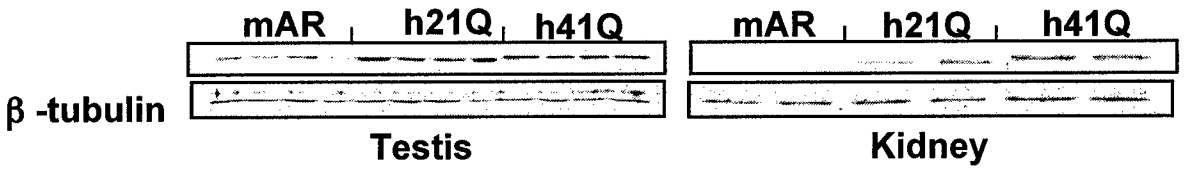

Figure 2 – AR protein levels in cell extracts from testis and kidney of wild type mice compared to mice with humanized 21Q or 41Q alleles. Each lane represents an individual mouse. Whole cell extracts were prepared by standard procedure and 10 μg (testis) or 30 μg (kidney) protein separated by SDS-PAGE. Gels were transferred to nylon membrane and exposed first with anti-AR antibody, and subsequently with anti-β tubulin as an internal control for quantitation.

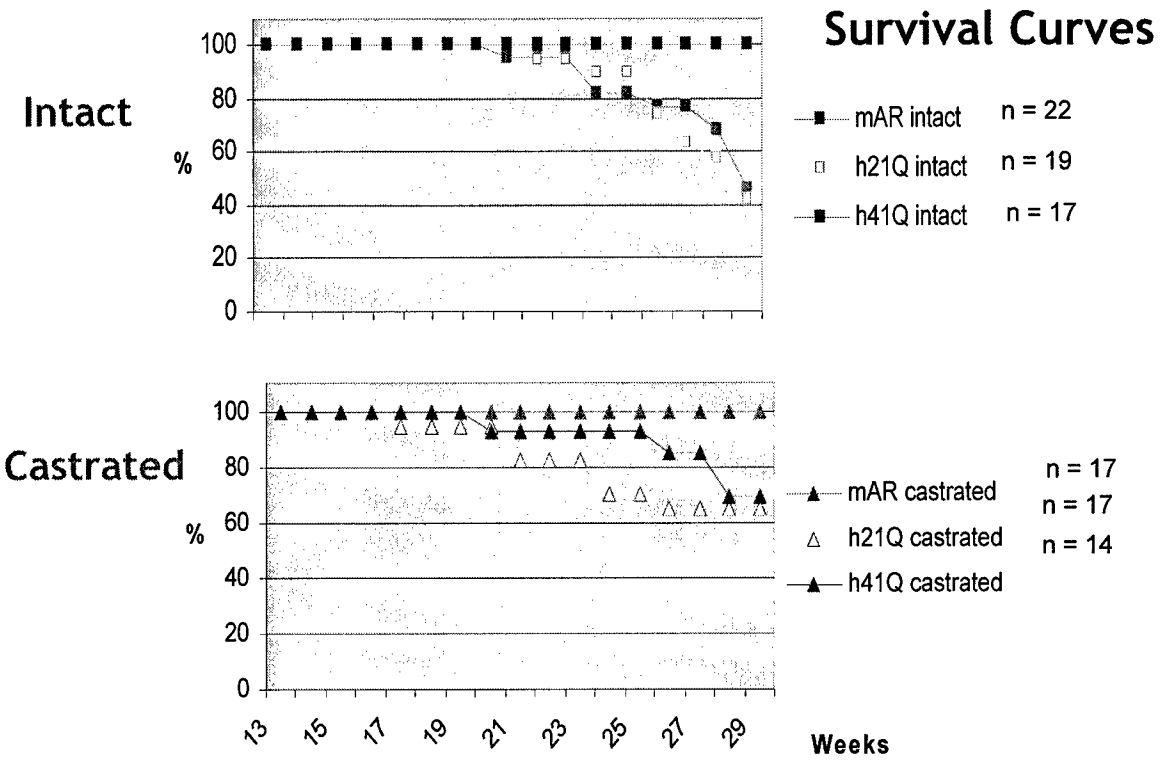

Figure 3 – Survival curves for TRAMP mice carrying wild type mAR, 21Q or 41Q humanized ARs. Intact or 12 week-castrated mice were aged, and euthanized when moribund, with tumors removed for analysis. 29 weeks is the arbitrary cut-off for this comparison (a complete time-course is being prepared with the additional mice). In intact mice, the long Q tract allele offers protection against tumor initiation, whereas in castrated mice, both humanized alleles permit earlier and more aggressive disease.

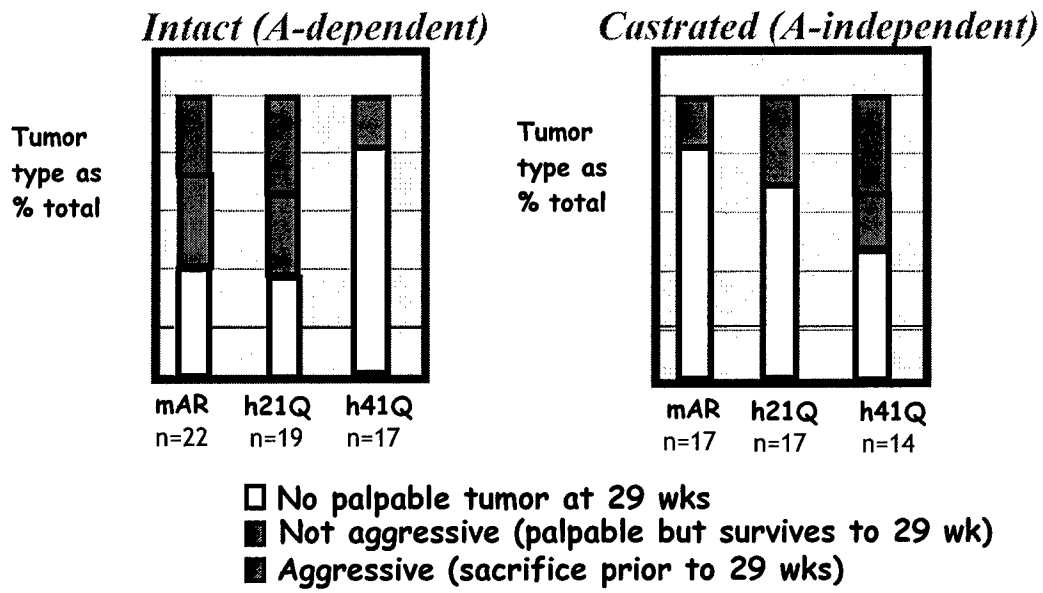

Figure 4 – Histogram comparison of tumor lethality, using palpation of tumor at 29 weeks as diagnostic. Aggressive tumors required mouse sacrifice prior to 29 wks, weakly aggressive were palpable at 29 wks but not moribund, and least aggressive disease showed no palpable tumor at 29 wks.

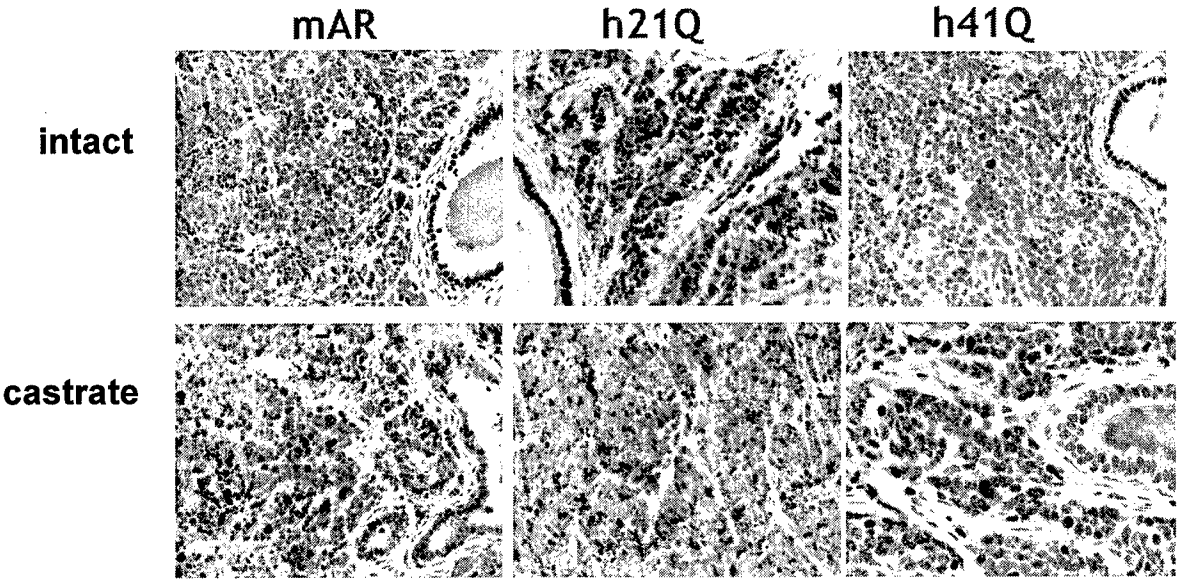

Figure 5 – Immunohistochemistry performed with an anti-AR antibody on paraffin-embedded prostate tumor samples from TRAMP mice, bearing either the wild type mouse androgen receptor (mAR) or humanized alleles with 21 or 41 glutamines in the Q-tract (h21Q, h41Q). Note in intact mice strong nuclear staining, especially for adjacent normal glandular tissue; the staining appears most intense for the 21Q but this must be analyzed over a large number of mice. Note also that in the tumor, the staining is heterogeneous – some cells are strongly positive and others not. In the castrated mice, staining is more diffuse, in accord with lack of hormone and therefore loss of nuclear localization. Interestingly, there is still strong nuclear staining in some cells of the 21Q castrate tumor, perhaps indicative of ligand-independent receptor activation. Staining is extremely low in the 41Q castrate.

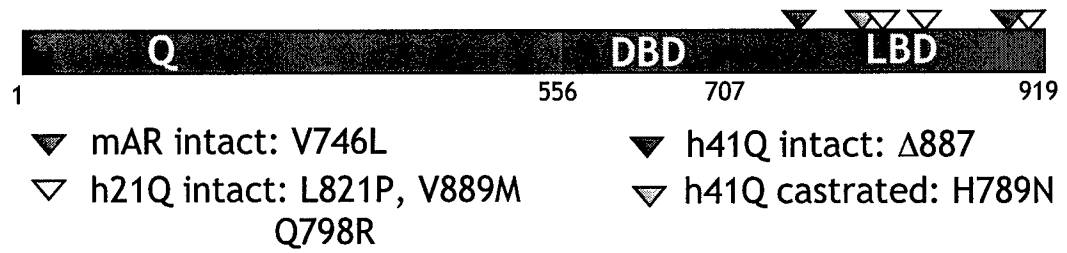

Figure 6 – 2-3 tumors of each genotype have been analyzed thus far for mutations in AR (excluding the N-terminus). Above are shown missense mutations that have been found; a similar number of silent mutations have been seen and are not shown above. The mutations found in the mAR tumors and 21Q h/mAR tumors have also been found previously in PCa or PAIS, but the 41Q h/mAR mutations are novel. As very few samples have been completely analyzed, conclusions are probably premature.