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14. ABSTRACT We proposed to study the mechanisms of androgen resistance by focusing on androgen receptor mutations and may arise due to selective pressures of antiandrogen treatment. We have utilized xenograft and a humanized mouse model of prostate cancer. To date we have identified mutations throughout the androgen receptor coding region and are in the process of carrying out functional analysis. It is proposed that these mutations may lead to greater androgen receptor activity under particular conditions and potentially highlight sites of interaction with critical co-factors that might themselves serve as novel therapeutic targets to complement androgen ablation. During this study analysis of tumor progression in the h/mAR X TRAMP mice have highlighted differences in disease course between antiandrogen treated and hormone deplete (castrated) mice. We are in the process of determining mechanisms underlying these differences by characterizing AR levels and subcellular localization in these tumors					
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Introduction

A major concern in the treatment of prostate cancer is the choice and timing of endocrine therapy. Androgens play a critical role in prostate growth and differentiation and this hormone dependence has been utilized extensively in the treatment of localized and advanced prostate cancer. While inhibiting androgen synthesis and/or blocking androgen receptor (AR) action initially slows tumor progression, androgen ablation inevitably fails. This is despite the continued presence of the androgen receptor and evidence that suggests that the androgen signaling pathway remains intact [1].

Suggested mechanisms of androgen resistance include androgen receptor amplification, ligand independent AR activation, androgen receptor mutation, as well as, aberrant activation of growth signaling pathways that are independent of androgen receptor action [2, 3, 4]. This study aims to address the role of androgen receptor mutation in the attainment of androgen resistance. We hypothesize that the selection of mutant ARs may be treatment specific, suggesting that the genetic alterations found in AR are a direct consequence of the specific endocrine selection pressures applied to the prostate tumor during androgen ablation therapy.

We proposed to identify AR variation in tumors excised from xenograft prostate cancer mouse models treated with the antiandrogens and to characterize each AR variant *in vitro* to assess functional significance of each AR mutation.

The specific aims outlined in the proposal were as follows:

1. To compare the mechanistic actions of bicalutamide and flutamide in prostate cancer cells *in vitro*.
2. To use PC-3 and VCaP xenograft mouse models to test the hypothesis that different treatment regimes (no treatment, bicalutamide, flutamide, in the presence and/or absence of hormone) selects for treatment-specific AR variants.
3. To characterize functionally AR molecular variants that may result from antiandrogen treatment of the mouse xenograft models and to test their capacity for tumor promotion.

Body

Xenograft Studies

As was highlighted in the 2005 annual report, early experimental results indicated that the xenograft model system of prostate cancer utilizing the PC3, PC3^{-hAR}, PC3^{-LNCaPAR} cell lines may be inappropriate for investigating the potential selection pressures of the antiandrogens *in vivo*. These cell lines once injected into nude mice failed to show hormonal response.

The VCap cell line did show hormonal response *in vivo* (See fig 1) with androgen withdrawal initially slowing xenograft growth and then exhibiting androgen independent growth. As detailed in our previous report, we analyzed VCap androgen receptor

variation and highlighted several mutations. In accordance with the statement of work we are currently characterizing some of these mutations *in vitro*.

We identified a 69bp insert from our VCaP xenografts. This AR mutation was of particular interest as it was also found in 5 of 8 antiandrogen treated human prostate cancer patients that were sequenced in another project of in our laboratory. This insertion results in an androgen receptor with a 23 extra amino acids between the two zinc fingers of the DNA binding domain. This insert has also been reported in a family with partial androgen insensitivity. Although *in vitro* analysis highlighted defective DNA binding and transcription [5], we considered that presence of this mutated androgen receptor within a cancer cell may influence AR protein–protein interactions and potentially influence AR repression. Previously it has been shown that activated AR can decrease TPA stimulated NFkB transactivation [6, 7]. To examine potential AR repression we examined the effect of the 69bp insert AR on NFkB reporter activity in the presence of TPA and testosterone. As is evident in Fig 2, the mutated AR was unable to repress NFkB activation.. Further studies are required to elucidate the potential effect of this mutated AR on prostate cancer progression.

The Humanized mouse model- h/mAR x TRAMP

With the realization that the xenograft mouse model offered limited opportunity to assess treatment specific generation of AR mutations we decided to utilize an alternative experimental strategy while maintaining the overall proposal objective. We focused our attention on an alternative mouse model of prostate cancer that was generated in our laboratory, the humanized androgen receptor mouse model of prostate cancer- h/mAR X TRAMP.

In the h/mAR X TRAMP mouse model the first exon of mAR was swapped with the equivalent portion of hAR, the region most divergent between the species, creating a mouse with a ‘human-like AR’ under the control of mouse regulatory sequences. [8]. Crossing this humanized (h/mAR) mouse strain to the transgenic adenocarcinoma of the mouse prostate (TRAMP) tumor model allowed us to investigate, *in vivo*, the human AR and prostate disease coupled in a homogeneous genetic background. Importantly work from the Greenberg laboratory illustrated that the TRAMP model was an appropriate tool to investigate the possible generation of treatment specific AR. This study showed that distinct androgen mutations were generated in intact and castrate mice indicating that AR variants can vary with hormonal status [9].

The h/mAR x TRAMP mice were treated with antiandrogens (flutamide, 25 mg/kg or bicalutamide, 50 mg/kg) at two distinct time points: 12 wks of age when TRAMP mice exhibit prostatic intraepithelial neoplasia (PIN), or later, at the time of first tumor detection (fig 3). Tumors were assessed by abdominal palpation weekly. Control groups included untreated mice and mice castrated at 12 wks of age. Once h/mAR x TRAMP mice became moribund, they were sacrificed and all tumors were excised. Half of the tumor mass was fixed in formalin and prepared for immunocytochemistry. cDNA was

generated from the remaining prostatic tumors and the androgen receptor was PCR amplified, subcloned, and 20 clones/ tumor sequenced.

To date at least 20 AR's have been sequenced from each tumor with 10 tumors assigned to untreated control, castrate, bicalutamide and flutamide treated groups. We are in the process of analyzing these mutations to highlight AR variants that may be of functional significance. Recurring mutations identified to date are highlighted in Fig X. It is interesting that these AR mutations are found throughout the AR coding region regardless of endocrine treatment. This contrasts with work from the Greenberg lab which highlighted that mutations found in intact mice clustered to the ligand binding domain of the androgen receptor while those identified in the hormone deplete animal were located to the n-terminal transactivation domain [9]. We did observe clustering of recurring mutations however, around the polyglutamine tract and the hinge region of the humanized AR.

We will continue to sequence the AR from the remaining h/mAR xTRAMP tumors and complete the mutation profiling. Once detailed analysis of AR mutations has been completed we hope to characterize some AR variants in vitro to assess functional significance.

The primary objective of this proposal was the identification of AR variants that could contribute to the development of androgen independent disease; however careful analysis of tumor progression in these mice also provided an insight into differential actions of specific hormonal therapy. Antiandrogen treatment at the sign of first detectable tumor models prostate cancer patient treatment, while treatment beginning at 12 weeks provides a model to test the influence of early adjuvant treatment has on survival.

Mice treated with bicalutamide or flutamide from initial tumor detection showed slower disease progression compared to untreated control mice, as expected (Fig 4). Interestingly, mice treated at 12 wks before palpable disease, showed no significant survival benefit. However treatment with antiandrogens versus complete androgen withdrawal (castration), both at 12 wks, revealed a significant difference in time with disease. In castrates, once a tumor was palpable, death followed within a few weeks, whereas tumors in mice treated with antiandrogens progressed more slowly. This suggests that AR is differentially affecting disease course dependent on whether its ligand binding pocket is unoccupied, as in castrates, or occupied by antiandrogen. AR levels, subcellular localization and alternative signaling pathways are being tested for mechanisms that may underlie these differences.

Key Research Accomplishments

- Development of VCap xenograft model that illustrates appropriate hormonal response.
- Identification of novel somatic AR mutations that are found in functionally important regions suggests that they may play a part in influencing prostate cancer disease progression.
- Utilization of h/mAR x TRAMP mouse model has highlighted differences between complete androgen withdrawal and antiandrogen treatment. This has direct significance for human patients.
- The humanized mouse provides a unique model of prostate cancer that could be utilized to test new drugs

Reportable outcomes

Data generated in this study has been presented at local and international meetings. Abstract details as follows;

O'Mahony, O.A., Albertelli, M.A., Cadillac, J.M. and Robins, D.M. Effects of Antiandrogens versus Androgen Withdrawal on Prostate Cancer Progression in a Mouse Model. ENDO 2006, Boston, US

Steinkamp, MP, **O'Mahony, OA**, Albertelli, M A., Brogley, M.A, Butler, T, Gerber, J, and Robins, DM. Profiling of Androgen receptor mutations in mouse and human prostate cancer. Endo 2007 Toronto

Conclusions;

This study has focused on profiling androgen receptor mutations in xenografts and a humanized mouse model. We propose to identify directly relevant sites in human AR, especially in the n-terminal domain where mouse and human AR are most divergent. We are also comparing mutations in tumors of h/mAR-TRAMP mice following different treatment regimens. Complete analysis and functional assessment of AR mutations is ongoing. It is proposed that these mutations may lead to greater AR activity under particular conditions (gain-of-function), and potentially highlight sites of interaction with critical cofactors that might themselves serve as novel therapeutic targets to complement androgen ablation.

The striking variation in disease course observed in the h/mAR-TRAMP mice dependent on level of hormone or presence of antagonist suggests that AR differentially affects disease according to both quantitative and qualitative differences of the ligand-occupied LBD. We are in the process of determining mechanisms underlying these differences by

characterizing AR levels, subcellular localization and alternative signaling pathways in these tumors.

References

- [1] Culig Z et al, *Endo Related Cancer*, 2005, 12(2), 229-44
- [2] Haapala K et al 2007, *Human Pathology*,38(3),474-8
- [3] Taplin ME et al, *N England J Med*, 1995 21, 1393-8
- [4] Reynolds AR et al *Br JPharmacol*.2006, 147 S2, S114-52
- [5] Bruggenwirth et al *Am.J.Hum.Genet*, 1997, 61:1067-1077
- [6] Jorma J. et al *J. Biol. Chem.* 271: 24151-24156.
- [7] Aarnisalo P et al *Endocrinology* 140: 3097-3105
- [8] Alberteli, M.A., et al *Mol Endo*, 2006 20(6), 1248-60
- [9] Han G et al,*J.Biol Chem*, 2001, 276(14), 11204-13.

APPENDIX

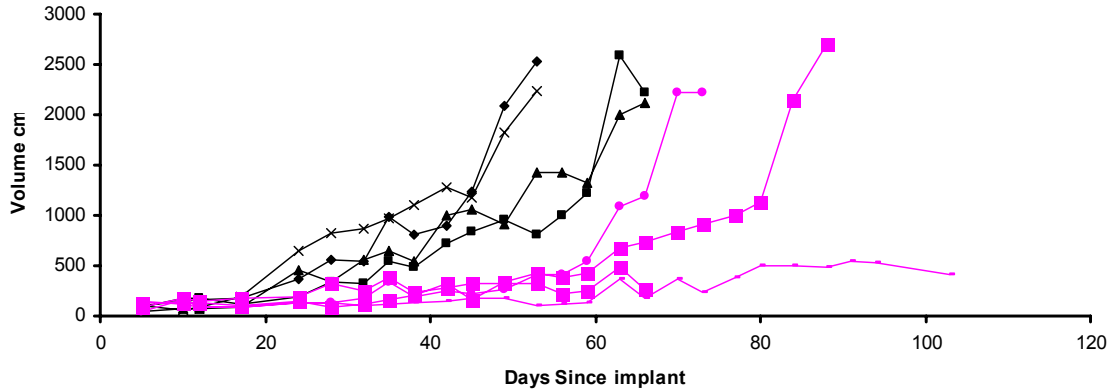


Figure 1: VCaP xenograft tumor growth. Pink lines represent castrated mice.

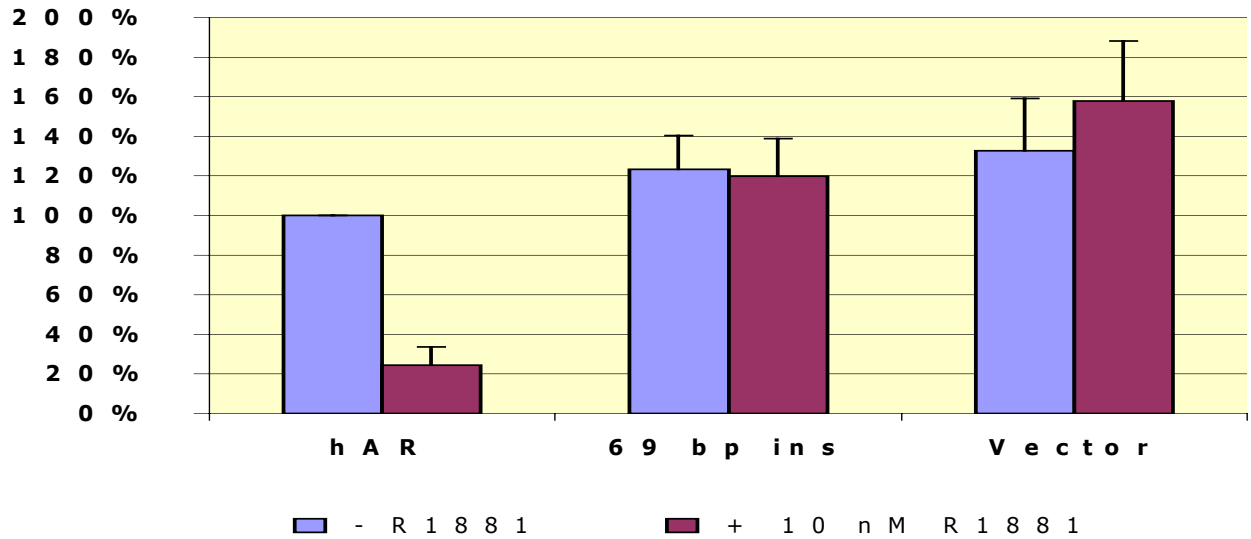


Figure 2-CV-1 cells were transfected with mutant or wild type AR and treated with 5 ng/ml TPA to induce NFkB expression and 10 nM R1881 to activate AR. The mutant 69bp insert was unable to repress NFkB transactivation. Data expressed as percent of wild-type activity.

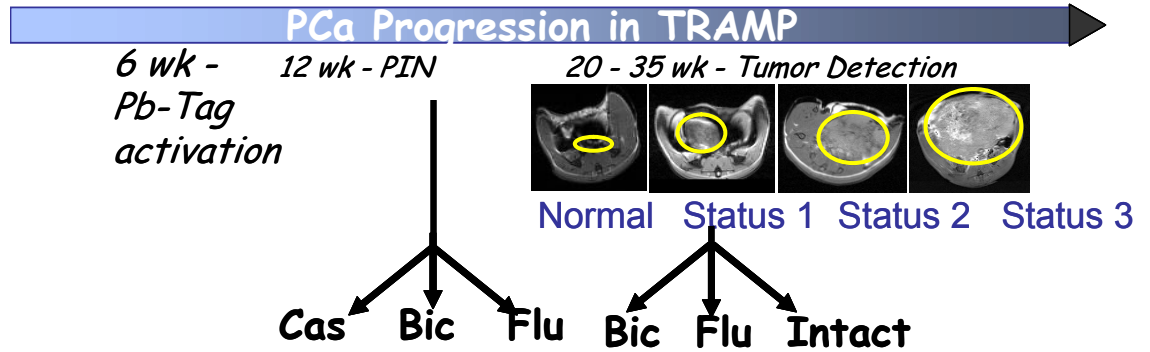


Figure 3- Mice were randomly assigned into 3 treatment groups, castration, bicalutamide, and flutamide. Mice were treated at two distinct time-points 12wks of age when prostatic intraepithelial neoplasia is evident and when tumors are first detected.

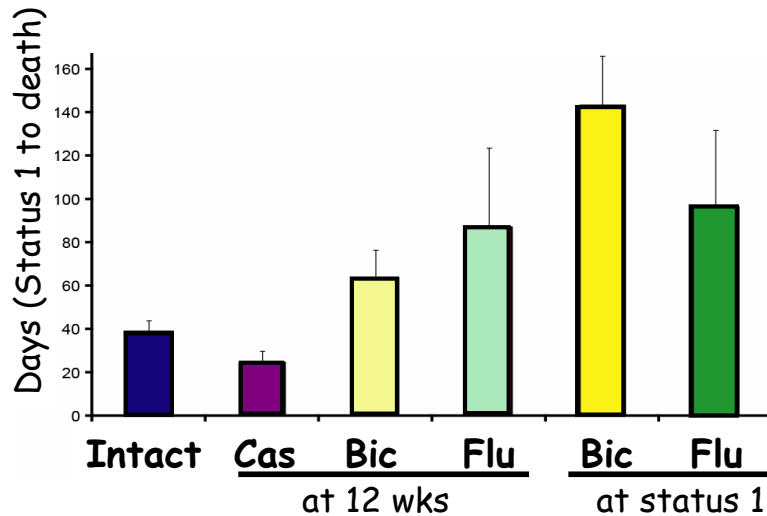


Figure-4 h/m ARx TRAMP mice were treated at two distinct time points, at 12 weeks of age and at the time of first detectable tumor growth. Early treatment does not offer a survival advantage. There is a significant difference between complete androgen withdrawal and antiandrogen treatment. This suggests that the AR is differentially affecting disease course, dependent on androgen concentration.

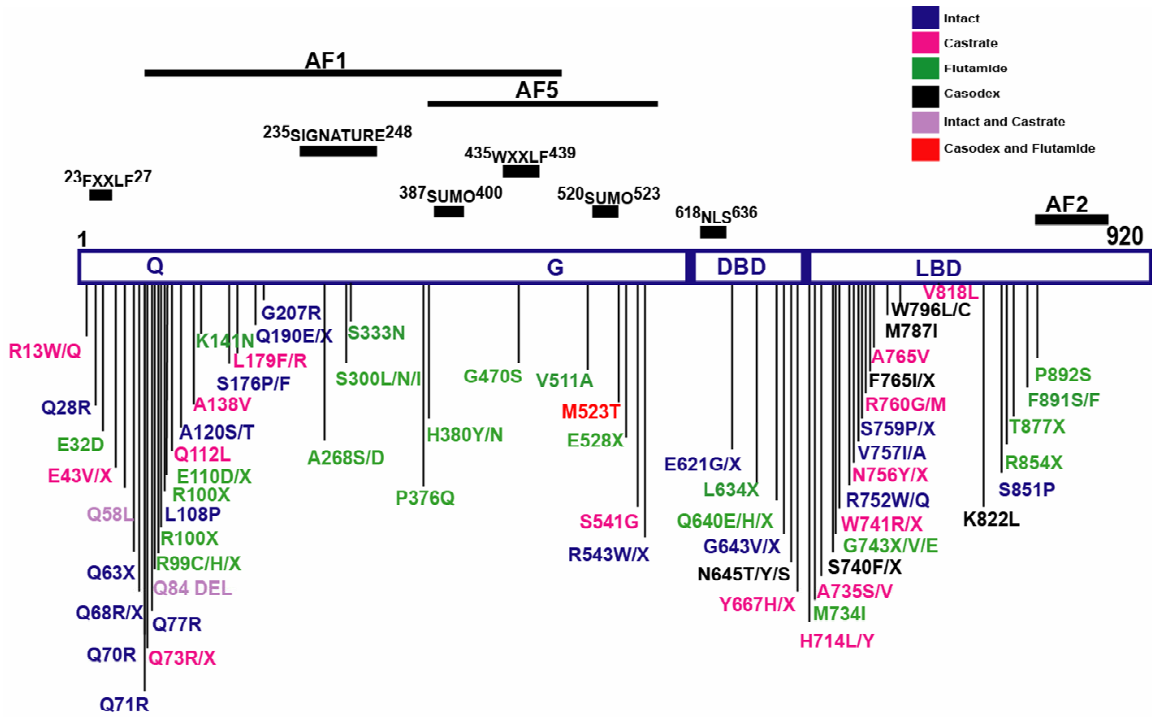


Figure-5- Mutations highlighted above reoccur in at least 2 tumors within each of treatment group.