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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. This provides a genetic paradigm in which to dissect AR functions that determine response to treatment. We are studying the role of the AR Q tract in ligand-independent AR activation in vitro and in a mouse model with prostate cancer ontogeny similar to human. In the mouse model, molecular correlates of differential response to castration will be determined using bioinformatic analysis of microdissected tumor samples. In the second year of this award, we have accrued the experimental mice from the strains constructed last year, using a modified genetic strategy thought to optimize similarity to human tumorigenesis. In the in vitro studies, we showed that ARs with different Q tract lengths are differentially responsive to signal transduction cascades likely to be influential under castrate conditions. We have tested several different pathways of such ligand-independent activation for their sensitivity to Q tract length. A phosphorylation site in the AR hinge region was examined by mutagenesis for a role in mechanisms underlying Q tract effects. Finally, we have begun to characterize differential prostate pathology in the mouse model as some animals have reached their designated end points.					
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Humanized Androgen Receptor Mice: A Genetic Model for Differential Response to Prostate Cancer Therapy

INTRODUCTION

Prostate cancer relies on androgen working via the androgen receptor (AR) for growth. Therapy therefore targets this signaling pathway and although initially successful, tumors ultimately become resistant to androgen ablation. In these castration-recurrent tumors, AR levels remain high and AR signaling persists, implying that disease is independent of androgen but remains dependent on AR (1). To examine the role of AR in human disease, we created a mouse model by germ-line gene targeting in which human AR sequences replace those of the mouse (2). Since shorter length of an N-terminal glutamine (Q) tract has been linked to PCa risk, we created alleles with 12, 21 or 48 Qs to test this association. These three “humanized” AR strains (h/mAR) are physiologically normal. However, upon activation of a prostate-targeted oncogene, distinct allele-dependent differences in initiation and progression of cancer are evident. These differences also impact progression following androgen ablation, with each allele conferring a distinct response (3). Mice with the transcriptionally more active 12QAR respond well to castration, whereas those with the weaker 48QAR show no benefit from treatment. These mice provide a genetic paradigm in which to dissect functions of AR that determine response to therapy. Identifying distinct pathways following treatment may reveal novel markers of response to therapy and suggest distinct strategies for optimal treatment. In this grant, we are studying the role of the Q tract in ligand-independent AR activation *in vitro* (Aim I) and, in a mouse model more similar to human cancer ontogeny we will determine molecular correlates of differential response to castration (Aim II). In Aim III, results from mice will be compared *in silico* to human datasets to identify pathways signifying differential response to therapy.

BODY

The grant outlined 4 tasks in the Statement of Work. We have completed the first using a modified genetic strategy, have made substantial progress on the second, and have accrued experimental animals necessary for the third with initial characterization of pathology. Relevant details, emphasizing those not previously reported, are described below.

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

We initially proposed conditional PTEN deletion (4) to initiate prostate tumorigenesis in C57BL/6 mice, for each h/mAR allele (12Q, 21Q, 48Q). Early in the project we modified the strategy since results of others suggested: a) the C57BL/6 background is resistant to PCa and FVB is preferable (5); b) conditional PTEN deletion is not uniform in the prostate, leading to potential difficulties interpreting data (6). Thus we bred the Q tract alleles onto the FVB background for 5 backcross generations, and modified the oncogenesis strategy to initiate cancer by global inactivation of one PTEN allele and cooperation of a prostate-targeted ETV1 transgene (7-9). This approach will provide more uniform oncogenesis than cre-mediated prostate-specific deletion of PTEN proposed before. Creation of the breeder strains differs, but overall mouse numbers and experimental design are similar subsequently. The modified procedure and animal approval was obtained from U-M’s UCUCA and from the DOD MRMC Animal Use Committee.

h/mAR mice were backcrossed onto the FVB background; all other mice needed were available on the FVB background. We created 3 strains doubly homozygous for one of the h/mAR alleles and carrying the ARR2PBi-ETV1 prostate-specific transgene. These mice were crossed to PTEN heterozygotes to generate experimental and control animals.

For each Q tract allele, 3 experimental groups (**n=8**) will be compared – **pretreatment** (12 weeks of age), **untreated** (intact at 24-40 weeks) and **treated** (castrated at 12 weeks, aged to 24-40 weeks). An equivalent number of littermate controls with one or both active PTEN alleles as well as controls lacking the ETV1 transgene, will be maintained. Upon reaching the time point (Task 3), prostates will be microdissected and processed for histological and molecular analysis.

For each Q tract allele, we have currently obtained at least 30 experimental and 30 control mice. Some have reached their time points and tissues have been harvested but many are still aging. The model is proving to be less aggressive than some reports have indicated and we have thus had to modify the time point to 10 rather than 6 months to insure sufficient evidence of malignancy.

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

The Q tract influence we noted in mouse PCa (3) suggests differences in AR strength may contribute to individual differences in refractory disease. Short Q tract ARs are transcriptionally more active at low androgen levels due to both greater intramolecular AR interaction between the N and C terminus (N/C interaction) and to greater coactivator recruitment (10). AR can also be activated in the absence of androgen by growth factors, particularly IGF, EGF, cytokines (IL-6), PKA and tyrosine kinase receptors such as Her2/neu (11, 12). In Task 2, we wish to test whether growth factor signaling pathways are also sensitive to Q tract length and thus underlie the action of the Q tract in castration-resistant prostate cancer. Task 2 aims to probe: **a)** cell-type and promoter-specific differential activation by transfected 12Q, 21Q and 48Q AR cDNAs in the presence of low or no hormone, and **b)** ligand-independent differential activation elicited by co-expression of a constitutively active growth factor, in low or no hormone. Our overall hypothesis is that shorter Q tract ARs are hypersensitive to low ligand and show greater activation by growth factors, perhaps by differential post-translational modification. Furthermore, this greater AR activity under castrate conditions promotes more differentiated and slower tumor growth.

As reported last year, to test central signal transduction pathways rather than specific growth factors, we proposed to use constitutively active MEKK1, but switched to constitutively active Raf for broader activation (13). We tested Q tract allele activity in RWPE-1 cells, immortalized normal prostate (14), and PC-3, the classic late-stage PCa cells. Both are human cell lines that do not express endogenous AR. For promoters, we tested the hallmark AR-responsive prostate-specific antigen (PSA) enhancer/promoter, which is a complex promoter with multiple binding sites for AR as well as other transcription factors (15). We also tested two distinct androgen responsive elements – the consensus inverted repeat that is also recognized by other steroid receptors (HRE3) and a related direct repeat that is more AR-specific in binding (HRE2) (16). The PSA promoter and multimerized HREs (3XHRE3, 4XHRE2) were linked to a minimal *tk* promoter driving a luciferase reporter gene for transient transfection, and sensitivity tested to the synthetic non-metabolizable androgen, R1881, at 0.01 and 1 nM concentrations, representing castrate and intact levels of androgen, respectively. Data presented last year showed that differences in AR activation that varied with Q tract length were significant in cancer but not

normal cell lines, and were pronounced for the AR binding sites alone but were masked in the complex PSA promoter, likely due to compensatory effects of additional factors. It is particularly interesting that the cancer cell background accentuates the Q tract effect; this could be due to differences in other transcription factors or levels of coactivators, which we plan to pursue in part by testing whether addition of specific coactivators (SRC-1, SRC-2, SRC-3) can enhance the modest differences demonstrated in this experiment, particularly at low ligand levels.

To test sensitivity of ligand-independent growth factor activation to Q tract length, we utilized a constitutively active Raf-1 kinase to activate the MAPK cascade (13). Constitutively active (ca) Raf-1 kinase is a serine-threonine kinase that contains only the catalytic subunit and lacks the regulatory subunits. As reported last year, in the absence of ligand, negligible activation of AR was obtained with (ca)Raf-1. However, in the presence of castrate levels of ligand (0.01 nM), (ca) Raf-1 enhanced activation of AR over ligand alone, and there was a trend for Raf activation to be inversely correlated with the AR Q tract length, with AR12Q showing the greatest additional activation by Raf in both PC-3 and RWPE-1 cells. This activation was greatest on the complex PSA promoter, suggesting Raf was influencing transcription factors additional to AR.

Raf-1 activation was modest, but at castrate levels of hormone AR activation was inversely correlated with Q tract length, as hypothesized (Fig. 1A). To determine whether other activators might accentuate this effect, we tested several other key signal transduction pathways reported to exert ligand-independent activation of AR for sensitivity to Q tract length. In particular, we used constitutively active versions of Rac1 and RhoA GTPases (17) (Fig. 1B). Interestingly, for these conditions, in contrast to Raf1, neither Rac1 nor RhoA enhanced AR activation at castrate levels of androgen. In fact, constitutively active Rac1 significantly inhibited AR activation. It is possible that activation might be seen at earlier time points since our transfection protocol differs from ones in which Rac1 activation was seen. In addition, the inhibition may reflect indirect

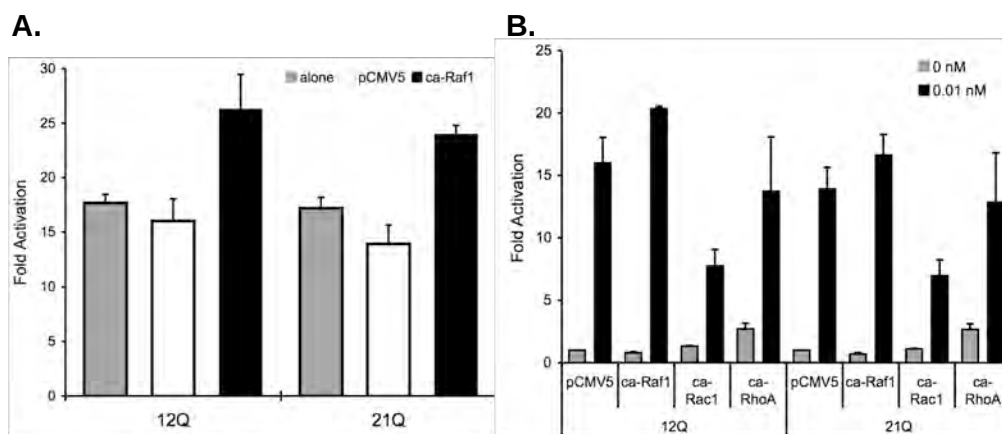


Fig. 1: (ca) Raf1-Kinase activates AR Q tract variants at castrate levels of androgen. Shown is the activation of the PSA promoter. Prior to transfection, PC-3 cells were fed phenol red free media and treated with either methanol vehicle or 0.01 nM R1881 for 4 hours. Cells were then transfected with 450 ng PSA reporter, 50 ng renilla internal control, 25 ng receptor alone (12Q or 21Q AR), and with either 25 ng of (ca) Raf1-Kinase or pCMV5 empty vector (A and B), or with 25 ng (ca) Rac1 or (ca) RhoA (B). 24 hours post transfection cells were refed media with vehicle or hormone for an additional 24 hours. The graphs represent relative luciferase activities normalized to renilla for 3 independent trials. Fold activation is calculated as activity induced by hormone over vehicle treated counterpart (A) or over vehicle treated empty vector (B).

effects on additional components and actors in the pathway, as well as direct effects on AR. Furthermore, it is intriguing to note that constitutively active RhoA, while not synergizing with low levels of hormone to activate AR, did promote AR activation in the complete absence of ligand, which has not been previously noted. These effects of Rac1 and RhoA, however, did not appear to be sensitive to Q tract length, unlike effects of ca-Raf1. Additionally, forskolin did not produce Q-tract-sensitive activation of AR (not shown). Cumulatively these observations help to direct our focus on mechanisms underlying Q tract sensitivity in AR ligand-independent activity.

Since growth factor signaling often elicits post-translational modifications to influence target protein activity, and signaling through the MAPK pathway induces AR phosphorylation (18), we examined the effect of activated Raf-1 kinase on phosphorylation of Q tract variant ARs. We reported last year our examination of the level of phosphorylation on serine residues at positions 81 and 650. Since S81 is adjacent to the Q tract, its phosphorylation might be influenced by length of the tract, which likely serves as a linker between functional AR domains (19). S650 is in the hinge domain of AR, which is involved in DNA binding and coregulator contact, and is phosphorylated in response to MAPK and other activators (20, 21). From Western blots with phospho-specific AR antibodies, presented last year, we found S650 to be more sensitive to ca-Raf1 phosphorylation than S81, and furthermore S650 phosphorylation was greater for 12Q and 21Q ARs. This suggested that Q tract length differentially impacted phosphorylation within the hinge domain of AR, perhaps due to the greater N/C interaction promoted by the short Q tract.

To probe the mechanism and outcome of this differential phosphorylation, we created ARs by site-directed mutagenesis that mimicked either constitutive phosphorylation at S650 (by a serine to glutamic acid [S → E] “phosphomimetic” amino acid substitution) or an inability to be phosphorylated (by an S → A change to a “phospho-dead” site) (Fig. 2A). These mutant ARs were transfected as before into PC-3 cells and reporter activation assessed; data for the consensus HRE reporter is shown in Fig. 2B. The phosphomimetic substitution (S650E) showed a pronounced influence on AR activity that was inversely correlated with Q tract length. This was most evident at intact levels of hormone but detectable also at castrate levels, particularly for the hypersensitive AR12Q. The AR48Q phosphomimetic mutant showed least effect relative to wild type. The non-phosphorylatable S650A mutants showed little difference from wild type 12Q or 21Q AR activity, but interestingly AR48Q activity was greatly decreased by this mutation.

This differential activity of Q tract variants dependent on phosphorylation at S650 is intriguing because phosphorylation at this site has been shown to influence AR subcellular localization rather than transactivation directly (21, 22). It is known that receptor export is important for recycling and reactivation (23). Moreover, the one “coactivator” shown so far to be sensitive to AR’s Q tract length proves to be RAN GTPase, which is involved in nuclear-cytoplasmic shuttling of numerous targets (24, 25). Thus, somewhat counter-intuitively, receptors that are exported more efficiently from the nucleus may achieve greater hormonal response, perhaps in part because they are more readily targeted by cytoplasmic signal transducers, and because even transient nuclear occupancy by receptor is sufficient for gene activation (23). The combination of phosphorylated S650 leading to rapid export and greater association with RAN allowing efficient nuclear reentry may account for the stronger activation in transfection by AR12Q.

In contrast to the phosphomimetic mutation, mutating S650 to alanine had little effect on 12Q or 21Q AR, but did reduce activity of 48Q by half relative to its wild type counterpart. This is

surprising since AR-S650A mutants are predominantly nuclear (21). To explore this further we investigated the subcellular localization of these receptors by immunocytochemistry (Fig. 2C). Despite the reduced activity in reporter gene assays, AR48Q-S650A displayed more nuclear localization at castrate levels of hormone compared to both AR48Q and AR48Q-S650E (note residual cytoplasmic staining for the latter two ARs). Further analysis of these AR mutants, in conjunction with Raf activation, may shed greater light on mechanisms underlying Q tract length effects on ligand-independent AR activation.

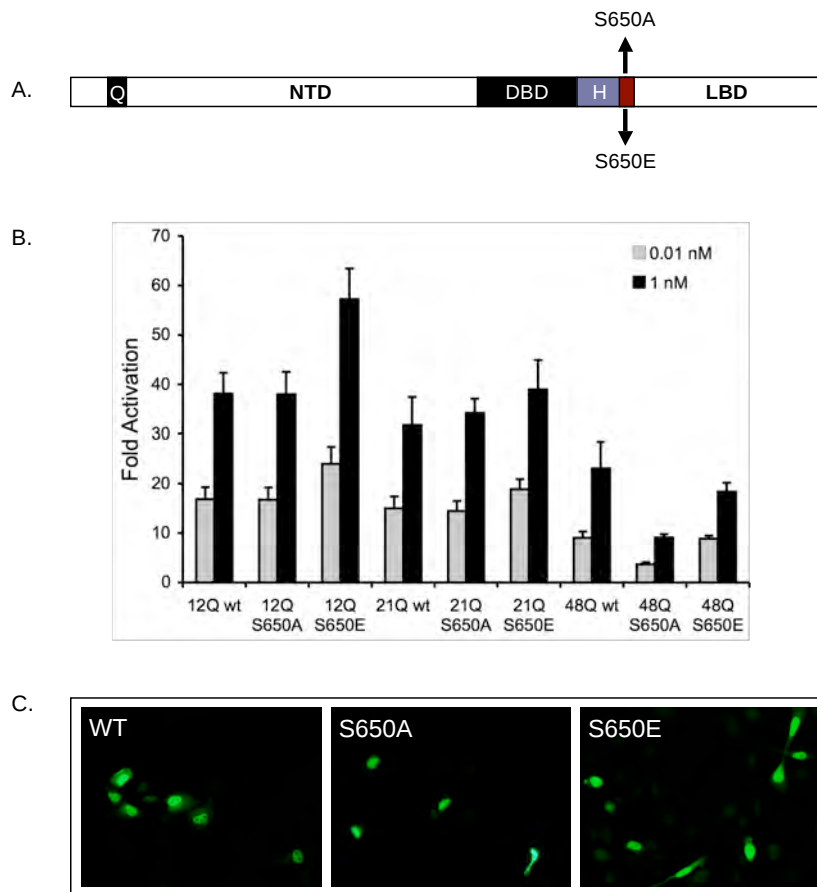


Fig. 2: Q tract length influences the activity of AR S650 phosphorylation mutants. Serine 650, a phosphorylation site in the hinge region of AR, influences AR transactivation and subcellular localization. **A.** Schematic of AR displaying the N-terminal transactivation domain (NTD) that contains the Q tract (Q), the DNA-binding domain (DBD) and the adjacent hinge region (H) that contains Serine 650 (S650), and the ligand-binding domain (LBD). Serine 650 was mutated to alanine or glutamic acid for these studies. **B.** Shown is the activation of the 3XHRE3-luciferase reporter gene by AR Serine 650 phosphorylation mutants. PC-3 cells were transfected with 400 ng 3XHRE3 reporter, 100 ng renilla internal control, and 4 ng receptor. 24 hours post transfection cells were fed with phenol red free media and treated with vehicle or hormone for 24 hours. The graph represents relative luciferase activities normalized to renilla for 4 independent trials. Fold activation is activity induced by hormone over vehicle treatment. **C.** Shown is the localization of the 48Q AR wildtype (WT), 48Q AR S-650 to alanine mutant (S650A) and the 48Q AR S-650 to glutamic acid mutant (S650E). PC-3 cells were seeded on chambered tissue culture slides and transfected 24 hours later with 100 ng receptor and 100 ng pCMV5 empty vector. 24 hours post transfection cells were fed phenol red free media and treated with 0.01 nM R1881 for 4 hours and fixed. AR detection used AR N20 primary antibody and FITC-conjugated secondary antibody.

Task 3. Determine the molecular correlates of androgen ablation response as affected by AR strength in prostate-specific PTEN-inactivated mice.

Part A of this task is to generate the experimental mice, and as mentioned above, all necessary animals have now been bred and have either reached their designated time point or are aging. We anticipate successful completion of this project but two issues will likely necessitate some additional time to completion and a request for a no-cost extension. First, while the AR12Q and AR48Q strains have bred well, there was a lag in generating AR21Q progeny and most of those animals are only a few months old. Second, our new model, relying for tumorigenesis on PTEN heterozygosity with a “second hit” provided by a prostate-targeted ETV1 transgene, proves to be slow, based on examination of a few pilot animals (see below) and we have thus extended the age of assessment from 6 months to 10 months. Because PTEN heterozygosity increases overall risk of cancer, about 25% of the mice are lost to lymphomas and other tumors; this has been accounted for by generating sufficient excess mice for analysis. The mice are examined at least every other day for palpable tumors so that diseased animals can be euthanized and prostate samples obtained even from mice sacrificed early. We are reluctant to extend the time point beyond 10 months due to more extensive losses. As shown below for a subset of pilot animals, by 10 months significant prostate abnormality as well as differences between Q tract lengths is evident. Even if we do not see more evidence of adenocarcinoma, we will still be able to analyze Q tract differences in prostate cancer initiation and response to early androgen ablation, with perhaps greater relevance to prevention more than to treatment.

Table I. Histopathology of 12Q vs. 48Q hAR Pten^{+/-} mice, +/- ETV1 transgene.

ETV1+			ETV1-			Status:			
12Q intact (n=2)			12Q intact (n=2)						
DLP	+	(1/2)	DLP	+	(2/2)		normal (-)		
VP	+, ++	(2/2)	VP	+, *	(2/2)		hyperplasia, modest (+)		
AP	+	(1/2)	AP	-			hyperplasia, significant (++)		
12Q castrate (n=2)			12Q castrate (n=3)				mPIN (*)		
DLP	+	(1/2)	DLP	-					
VP	-		VP	-					
AP	-		AP	-					
48Q intact (n=5)			48Q intact (n=2)						
DLP	+, ++, *	(4/5)	DLP	-			Prostate Lobes:		
VP	+, *	(3/5)	VP	+	(1/2)		DLP – dorsolateral		
AP	++	(1/5)	AP	-			VP – ventral		
48Q castrate (n=5)			48Q castrate (n=1)				AP – anterior		
DLP	+	(1/5)	DLP	+					
VP	+, ++	(2/5)	VP	-					
AP	-		AP	-					

Prostates were microdissected, fixed, sectioned and H&E stained slides examined by our pathologist, Dr. Rohit Mehra (Table I). Although only a few specimens per group were ready for analysis, several observations are encouraging: 1) the ETV1 transgene increases the extent of hyperplasia and abnormality across groups; 2) androgen ablation decreases hyperplasia for the 12Q more than for the 48Q group, as predicted by our earlier results in which the 12Q AR provided good response to therapy; 3) although it appears that there is more hyperplasia in the 48Q intact group, this may change with greater numbers and more in depth pathological analysis. These differences will be pursued by immunocytochemistry for key markers, and ultimately by obtaining RNA for quantitative analysis of gene expression, which is the goal of Task 4.

KEY RESEARCH ACCOMPLISHMENTS

- The Q tract variant h/mAR experimental mice have been derived on the C57BL/6 background, utilizing a new model for tumorigenesis that relies on PTEN heterozygosity and the prostate-targeted ARR2PBi-ETV1 transgene.
- 12Q, 21Q and 48Q ARs are differentially responsive in a promoter-dependent manner to some but not all signal transduction cascades likely to be operating under castrate conditions.
- Phosphorylation of AR by growth factor activation, particularly at Ser650, appears to correlate inversely with length of the Q tract, and may act by influencing subcellular localization of receptor.
- Preliminary analysis of a few mice suggests this model will be informative – the ETV1 transgene has a demonstrable effect on hyperplasia, and there are differences due to Q tract allele both before and after castration, as predicted by our analysis of TRAMP mice.

REPORTABLE OUTCOMES

We are extending the in vitro analysis of Q tract effects in ligand-independent AR activation and expect to publish these results within a year. I have discussed some of the preliminary results and approaches in a seminar presented at the University of Miami, Department of Molecular and Cellular Pharmacology (6/24/09).

CONCLUSION

In the first two years of this DOD IDEA award, we have constructed the appropriate mouse strains to establish a unique mouse model in which to address the role of the AR Q tract in differential response to androgen ablation. This model is optimized to circumvent previous problems due to strain background and lack of homogeneity in prostate cells, and utilizes a genetic paradigm more similar to human prostate cancer ontogeny (heterozygosity for PTEN and overexpression of ETV1). Experimental mice have been generated and prostates are being microdissected as animals reach their time points. Initial pathological analysis indicates that while tumorigenesis is slower than expected, AR Q tract differences are evident in intact as well as castrated mice. This suggests that bioinformatic analysis of gene expression will be informative in distinguishing markers of good versus poor response to castration therapy.

We have performed in vitro assays to examine molecular mechanisms underlying differential Q tract effects. ARs with different Q tract lengths are sensitive to promoter and cell type differences; this may impact diverging activities of AR during tumor progression dependent on Q tract length. Furthermore, we have shown that ARs with different Q tract lengths are differentially sensitive to growth factor signaling via the Raf1 pathway, demonstrated at both the level of transactivation in reporter assays and for phosphorylation of specific serines within AR. The means by which differential phosphorylation may impact AR activity may have more to do with differential nuclear localization (due to effects on both import and export) than to direct effects on transactivation. Subtle differences conferred by Q tract length may prove to be one of many factors that sum to significant affects in response to therapy.

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