TSPYL Family Regulates CYP17A1 and CYP3A4 Expression: Potential Mechanism Contributing to Abiraterone Response in Metastatic Castration-Resistant Prostate Cancer

Sisi Qin[1], Duan Liu[1], Manish Kohli[2], Liguo Wang[3], Peter T. Vedell[3], David W. Hillman[3], Nifang Niu[1], Jia Yu[1], Richard M. Weinshilboum[1] and Liewei Wang[1]

The testis-specific Y-encoded-like protein (*TSPYL*) gene family includes *TSPYL1* to *TSPYL6*. We previously reported that TSPYL5 regulates cytochrome P450 (CYP) 19A1 expression. Here we show that TSPYLs, especially TSPYL 1, 2, and 4, can regulate the expression of many CYP genes, including CYP17A1, a key enzyme in androgen biosynthesis, and CYP3A4, an enzyme that catalyzes the metabolism of abiraterone, a CYP17 inhibitor. Furthermore, a common *TSPYL1* single nucleotide polymorphism (SNP), rs3828743 (G/A) (Pro62Ser), abolishes TSPYL1's ability to suppress CYP3A4 expression, resulting in reduced abiraterone concentrations and increased cell proliferation. Data from a prospective clinical trial of 87 metastatic castration-resistant prostate cancer patients treated with abiraterone acetate/prednisone showed that the variant SNP genotype (A) was significantly associated with worse response and progression-free survival. In summary, *TSPYL* genes are novel CYP gene transcription regulators, and genetic alteration within these genes significantly influences response to drug therapy through transcriptional regulation of CYP450 genes.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ CYPs are involved in both drug metabolism and hormone biosynthesis. Variation in CYPs contributes to interindividual variation in drug response. However, a significant proportion of variation in CYP expression and activity remains unexplained by known *CYP* SNPs, indicating that additional mechanisms such as transcription regulation might be involved.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This study identified and characterized a new family of CYP transcriptional regulators, TSPYLs.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ TSPYLs transcriptionally regulate many CYPs. In the case of CYP3A4 and CYP17A1, TSPYLs' influence on the expression of these genes significantly alters response to abiraterone, a first-line treatment for CRPC, as we have demonstrated by both *in vitro* and patient association studies.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

☑ Our study has enhanced knowledge of genetic factors contributing to interindividual variation in drug response and it creates a foundation for future integration of both known polymorphisms in *CYPs* as well as well as additional genetic variants in *TSPYLs* to predict variation in drug response.

The testis-specific Y-encoded-like proteins (TSPYLs) have significant homology to the testis-specific Y-encoded protein (*TSPY*).[1] The TSPYL family includes six members, *TSPYL1* to *TSPYL6*. Most *TSPYL* family members are expressed in all human tissues based on Genotype-Tissue Expression (GTEx) datasets.[2] Except for *TSPYL3,* a pseudogene, *TSPYL* genes encode proteins that include a common C-terminal nucleosome assembly protein[3] domain that may function in chromatin remodeling and transcriptional regulation.[4,5] TSPYLs are known to be involved in many cellular functions,[6–8] and genetic polymorphisms, mutations, and/or methylation status for *TSPYL* genes are associated with a variety of diseases.[9–11] *TSPYL* genes, with the exception of *TSPYL2*, lack introns, possibly as a result of ancient retroposition events,[1] suggesting constitutive biological functions for these genes.

Our previous genome-wide association study (GWAS) of plasma estradiol concentrations in 772 postmenopausal women with estrogen receptor (ER)-positive breast cancer identified a genome-wide significant single-nucleotide polymorphism (SNP) signal within the *TSPYL5* gene (P = 3.49E-08).[12] Subsequent functional studies demonstrated that TSPYL5 regulates the expression of CYP19A1,[12] the aromatase that catalyzes the

[1]Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota, USA; [2]Department of Oncology, Mayo Clinic, Rochester, Minnesota, USA; [3]Department of Health Sciences, Mayo Clinic, Rochester, Minnesota, USA. Correspondence: Liewei Wang (Wang.Liewei@mayo.edu)

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synthesis of estrone and estradiol from androstenedione and testosterone, respectively.[13] In a role similar to that of CYP19A1 in estrogen synthesis, CYP17A1 is responsible for the synthesis of dehydroepiandrosterone (DHEA) and androstenedione,[14] precursors of testosterone.[15,16] CYP17A1 is mainly expressed in the adrenal gland and in prostatic tumor tissue, mediating the synthesis of both circulating adrenal and *de novo* intratumor androgens that can activate androgen receptor (AR) to promote prostate cancer growth despite achieving clinical castration.[17,18] Recently, a CYP17A1 inhibitor, abiraterone acetate (AA), in combination with prednisone demonstrated increasing survival in metastatic castration-resistant prostate cancer (mCRPC).[19] AA is hydrolyzed by esterases *in vivo* to the active metabolite, abiraterone.[19]

Both CYP19A1 and CYP17A1 are members of the cytochrome P450 superfamily, which consists of 43 subfamilies.[20] CYP1, 2, and 3 subfamilies are the major drug-metabolizing enzymes and are responsible for the biotransformation of 70–80% of exogenous substances.[21] Drug-metabolizing CYPs are predominantly expressed in liver. CYP3A4 is the most abundant CYP isoform and metabolizes more than 50% of drugs that are primarily cleared by metabolism,[22] including abiraterone.[23] A majority of abiraterone is eliminated in the feces either as unchanged prodrug, AA (55%), or the metabolite, abiraterone (22%), while 5% of abiraterone was excreted via urine as N-oxide abiraterone sulfate, which was oxidized by CYP3A4.[24] The inactive metabolites of abiraterone lose its inhibitory activity of CYP17A1.[23]

Since CYP and TSPYL family members are structurally and functionally highly similar, based on our previous findings[12] we hypothesized that TSPYLs might regulate the expression of many CYPs. In the present study we demonstrate that TSPYL1, 2, and 4 function as transcriptional regulators for various CYPs including CYP17A1 and CYP3A4. We also show that genetic variation in *TSPYL1* can influence the response to abiraterone. These observations indicate that TSPYLs can be a major source of inter-individual variation in drug response through the transcriptional regulation of *CYP* genes.

RESULTS

TSPYLs regulate CYP expression

To test the hypothesis that TSPYL proteins might regulate the expression of multiple CYPs, we first determined the mRNA levels of a series of CYPs related to steroid-biosynthesis and drug-metabolism after knocking down or overexpressing TSPYL1, 2, 4, and 5 in a human hepatoma cell line, HepG2. *TSPYL3* is a pseudogene and *TSPYL6* is expressed exclusively in testis (GTEx dataset) and it was not detectable by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in HepG2 cells. As a result, these two *TSPYLs* were not included. Among the CYPs tested, CYP2C9, CYP2C19, and CYP3A4 showed significantly increased mRNA levels, while CYP17A1 showed dramatically decreased levels after knocking down TSPYL1, TSPYL2, and TSPYL4 using two different siRNAs. Overexpressing TSPYLs confirmed those results (**Supplemental Figure S1**). TSPYL5 only affected the CYP19A1 mRNA level (data not shown), consistent with our previous findings.[12]

Since the endogenous levels of the CYPs are low in HepG2 cells, we further confirmed the results in HepaRG and NCI-H295R, an adrenal corticocarcinoma cell line. HepaRG cells are terminally differentiated hepatic cells derived from a human hepatic progenitor cell line that retains many characteristics of primary human hepatocytes.[25] The NCI-H295R cells have very high expression of the steroid-biosynthesizing CYPs, including CYP17A1.[26] We observed the same phenotypic changes in HepaRG cells as those seen in HepG2, including both mRNA (**Figure 1a**, **left**) and protein levels (**Figure 1a**, **right**). Knockdown results in HepaRG were confirmed with overexpression (**Figure 1b**). In NCI-H295R cells, except for CYP2C9, which was undetectable in this cell line, CYP17A1, CYP3A4, and CYP2C19 mRNA and protein levels were altered in the same directions as those observed in the hepatic cell lines (**Supplemental Figure S2**).

It has been suggested that castration-resistant prostate cancer not only takes advantage of circulating DHEA generated from the adrenal glands, but are also able to make intratumor androgens from cholesterol by upregulating CYP17A1.[27,28] Furthermore, CYP3A4 is detectable in prostate cancers.[29,30] Therefore, we set out to test the effect of the three TSPYLs on the expressions of CYP17A1 and CYP3A4 in prostate cancer cell lines. Two prostate cancer cell lines, LNCaP, a hormonally sensitive cell line, and 22Rv1, a castration-resistant line, were used for these experiments. Knocking down TSPYL1, 2, or 4 decreased CYP17A1 and increased CYP3A4 protein levels, even though the endogenous levels of CYP17A1 and CYP3A4 were much lower in these two prostate cancer cells than in HepaRG or NCI-H295R cells. Overexpression of these three TSPYLs resulted in decreased CYP3A4 and increased CYP17A1 (**Supplemental Figure S3**). Based on these results, we concluded that TSPYL1, 2, and 4 negatively regulate the expression of CYP3A4, CYP2C9, and CYP2C19 mRNA but positively regulate CYP17A1.

TSPYL1, TSPYL2, and TSPYL4 are transcriptional regulators for CYP2C9, CYP2C19, CYP3A4, and CYP17A1

To determine whether the three TSPYLs that influenced CYP expression might function as transcription factors binding to specific DNA sequences in *CYP* gene promoter regions, we performed chromatin immunoprecipitation (ChIP) assays with TSPYL1, TSPYL2, and TSPYL4 antibodies, respectively. HepaRG cells were used for ChIP assay because of their high endogenous protein levels for all three TSPYLs. A series of qPCR primers were designed to amplify the promoter regions of *CYP2C9*, *CYP2C19*, *CYP3A4*, and *CYP17A1* (**Supplemental Table S1**). The ChIP assay showed that all three TSPYLs bound to DNA sequences in the promoter regions of all four *CYPs* (**Supplemental Figure S4**). For each *CYP* promoter, the same DNA fragment was enriched by individual TSPYL antibodies, indicating that all three TSPYLs might bind to the same DNA sequence motif. For instance, DNA fragments that could be amplified by *CYP2C9* P6 and P7 primer pairs were enriched in all three ChIP assays (**Supplemental Figure S4a**). We then used a motif-based sequence analysis tool, MEME Suite, to identify the possible binding motif for these three TSPYLs. A DNA

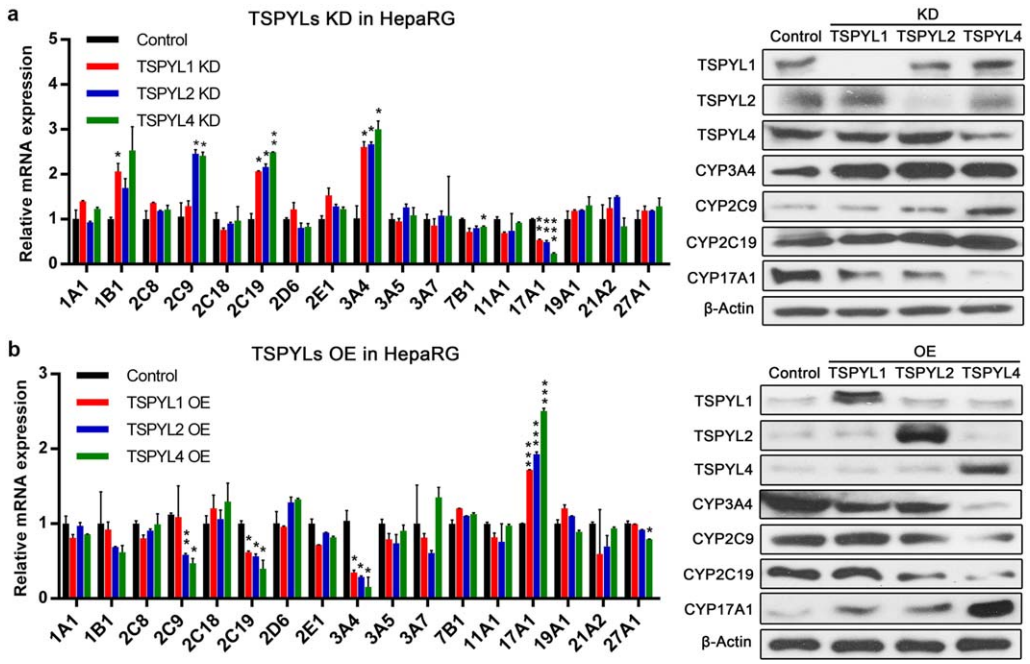

Figure 1 The expression of cytochrome P450s (CYPs) after knockdown or overexpression of TSPYL1, TSPYL2, and TSPYL4 in HepaRG human hepatic cells. (**a**) Knockdown studies of TSPYLs in HepaRG cells. (**b**) Overexpression studies of TSPYLs in HepaRG cells. mRNA expression levels relative to ACTIN are shown as means of three independent experiment (±SEM); expression levels of CYPs in these cells after knockdown or overexpression of TSPYLs were compared to cells transfected with negative siRNA or empty vector by the use of two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Changes of mRNA levels for CYP2C9, CYP2C19, CYP3A4, and CYP17A1 were validated by western blot analysis for HepaRG cells. Only mRNA results for CYP3A4 and CYP17A1 were validated in NCI-H295R cells since endogenous CYP2C9 and CYP2C19 levels were either undetectable or very low in this cell line (right).

sequence motif of CTXTGAGXTGTGTGGXXCCXACA GGXGTCCTGTXCTCCCAGXGXCTCXCT (best fit) with an *E*-value of 2.5E-08 was identified (**Supplemental Figure S5**). These ChIP assays provided evidence that TSPYL1, TSPYL2, and TSPYL4 are transcription factors for CYP2C9, CYP2C19, CYP3A4, and CYP17A1.

TSPYLs affect DHEA concentrations and cell proliferations

To determine whether the regulation of CYP17A1 expression by TSPYL1, TSPYL2, and TSPYL4 could influence DHEA biosynthesis, a process catalyzed by CYP17A1, we measured DHEA concentrations in NCI-H295R after knocking down the three TSPYLs individually or combined. CYP17A1 mRNA decreased more than 50% (**Supplemental Figure S6a**). As a result, DHEA concentrations in NCI-H295R cells also decreased significantly (**Supplemental Figure S6b**).

We further confirmed these findings in LNCaP and 22Rv1 cells. Downregulation of the three TSPYLs significantly decreased DHEA levels, consistent with the decrease in CYP17A1 mRNA levels in both LNCaP (**Figure 2a,b**) and 22Rv1 cells (**Figure 2e,f**). In addition, mRNA level for prostate-specific antigen (PSA), an indicator of DHEA function and androgen receptor (AR) activity, was also dramatically decreased (**Figure 2c,g**). Most important, knockdown of the TSPYLs resulted in an almost 30% reduction in cell proliferation for both prostate cancer cells (**Figure 2d,h**). Conversely, overexpression of the TSPYLs increased cell proliferation (**Supplemental Figure S7**). These results suggested that TSPYL1, TSPYL2, and TSPYL4 regulate CYP17A1 expression and, as a result, modulate the biosynthesis of DHEA and AR activation, affecting the proliferation of prostate cancer cells.

Abiraterone metabolism and response is regulated by TSPYLs

Abiraterone is the first and only FDA-approved CYP17A1 inhibitor that, in combination with prednisone, is used for the treatment of mCRPC. Abiraterone is metabolized by CYP3A4 to generate inactive N-oxidized metabolites. The three TSPYLs can induce CYP17A1 expression, resulting in increased DHEA levels and increased cell proliferation. At the same time, they also suppress CYP3A4 expression, potentially decreasing abiraterone biotransformation. Those observations stimulated us to test the effect of TSPYLs on abiraterone metabolism and anticancer activity. We first tested the effect of TSPYLs on response to abiraterone in prostate cancer cells. As expected, abiraterone significantly inhibited cell proliferation by inhibiting CYP17A1. Downregulation of TSPYLs reduced cell proliferation, probably as a result of reduced CYP17A1 expression. Therefore, knocking down TSPYLs did not further alter abiraterone response (**Figure 3c,f**). Downregulation of TSPYLs also increased CYP3A4 expression, causing reduced parent drug concentrations in LNCaP, 22Rv1, NCI-H295R, and HepaRG cells (**Figure 3a,b,d,e**; **Supplemental Figures S6c and d, S8e**). To further validate this observation, itraconazole (ITZ), a specific CYP3A4 inhibitor, was used to treat the cells during abiraterone treatment. Addition of ITZ restored the intracellular abiraterone

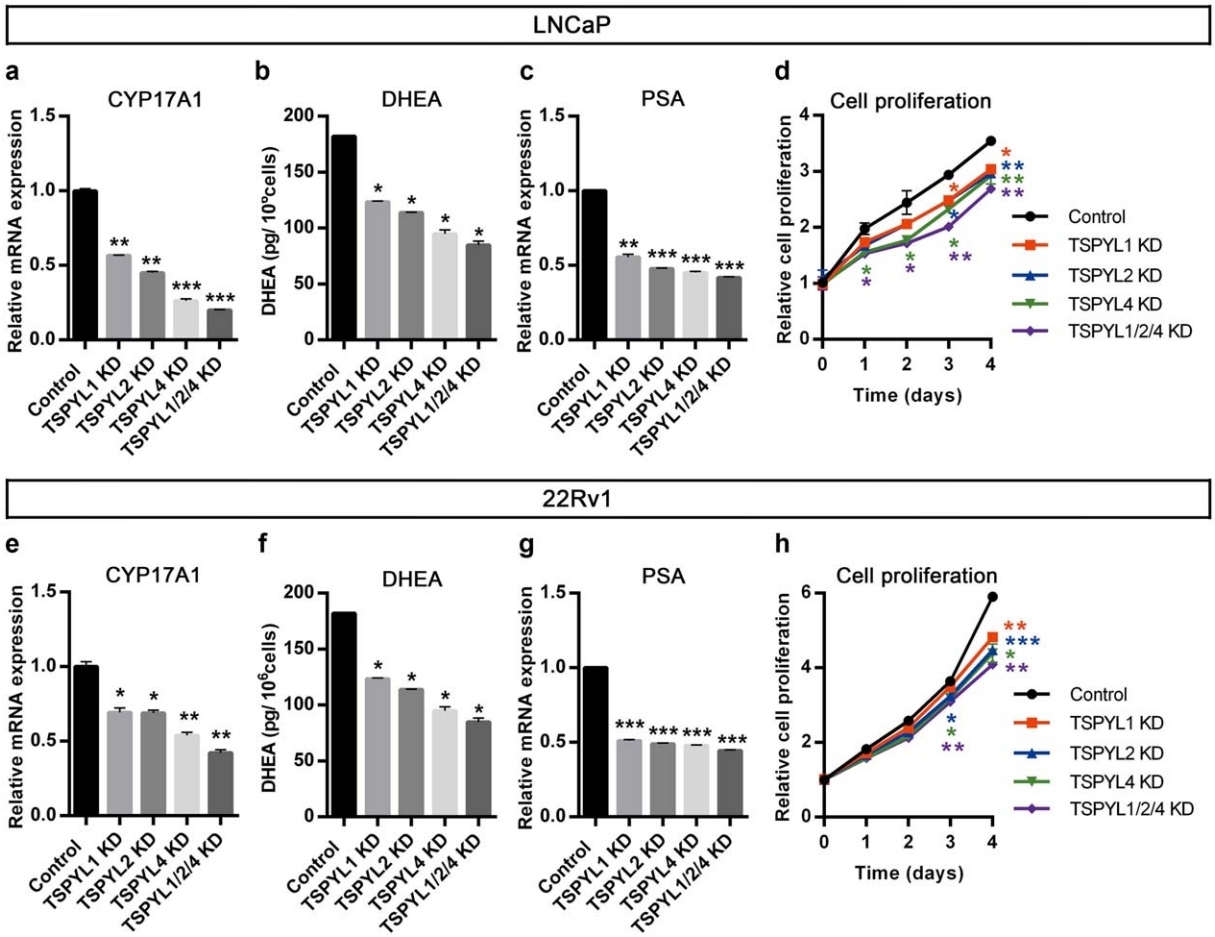

Figure 2 Changes of *CYP17A1* expression, DHEA levels, *PSA* expression, and cell proliferation after knockdown of TSPYL1, TSPYL2, and TSPYL4 individually or all three together in prostate cancer cells. LNCaP and 22Rv1 cells were cultured in 5% charcoal-stripped media for 48 h before transfection. After transfection, cells continued to be cultured in this hormone-free media plus pregnenolone at a physiological concentration (2 nM). Data are shown as mean ±SEM for three independent experiments. Forty-eight hours after transfection, the mRNA levels relative to ACTIN were determined by qRT-PCR and DHEA levels were determined with Enzyme-linked immunosorbent assay (ELISA). Cell proliferation after transfection was determined by MTS assay. The proliferation of cells after TSPYL knockdown was compared to that of cells transfected with a negative siRNA control at each timepoint by two-tailed Student's *t*-test and significance is indicated as asterisks with corresponding colors: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

level resulting from downregulation of TSPYLs (**Figure 3b,e**; **Supplemental Figures S6d and S8e**). ITZ, unlike another specific CYP3A4 inhibitor, ketoconazole, has no inhibitory effect on CYP17A1 activity even at a higher dose. A low concentration of ITZ alone had no effect on cell proliferation. However, in cells with TSPYL knockdown, ITZ together with abiraterone dramatically inhibited cell proliferation when compared with abiraterone alone (**Figure 3c,f**). In addition, TSPYLs did not affect the expression of SULT2A1 (data not shown). These results suggested that TSPYLs affect abiraterone response by regulating the expressions of both CYP17A1 and CYP3A4, the major abiraterone-metabolizing enzyme.

Functional characterization of *TSPYL1* and *TSPYL4* nsSNPs

TSPYL1, TSPYL2, and TSPYL4 transcriptionally regulate CYPs and influence drug response. We next determined whether any known nsSNPs in these three *TSPYL* genes might influence their function and/or affect drug response. Common nsSNPs with MAF values ≥0.10, as reported by the 1000 Genomes Project, were selected for study (**Supplemental Tables S2 and S3**). Four nsSNPs in *TSPYL1* and two nsSNPs in *TSPYL4* met that criteria and were used for functional studies. Variant expression constructs containing each of these six SNPs, together with wildtype (WT) constructs, were transfected into HepaRG, NCI-H295R, LnCaP, and 22Rv1 cells, respectively.

In HepaRG cells overexpressing the WT and variant constructs, none of the nsSNPs in either *TSPYL1* or *TSPYL4* displayed significant effects on TSPYL1 or TSPYL4 protein levels (**Supplemental Figure S8a,c**). However, one *TSPYL1* nsSNP, rs3828743, that resulted in a P62S amino acid substitution, abolished TSPYL1's suppression of CYP2C19 and CYP3A4 transcription, while the effect of TSPYL1 on the induction of CYP17A1 was not influenced by this SNP. Consistently, ChIP assays performed with TSPYL1 antibody using lysates from cells overexpressing TSPYL1 WT and variant allozymes showed that TSPYL1P62S resulted in loss of TSPYL1 binding to the promoters of *CYP2C19* and *CYP3A4,* but not *CYP2C9* and *CYP17A1* (**Supplemental Figure S8b**). Neither of the *TSPYL4*

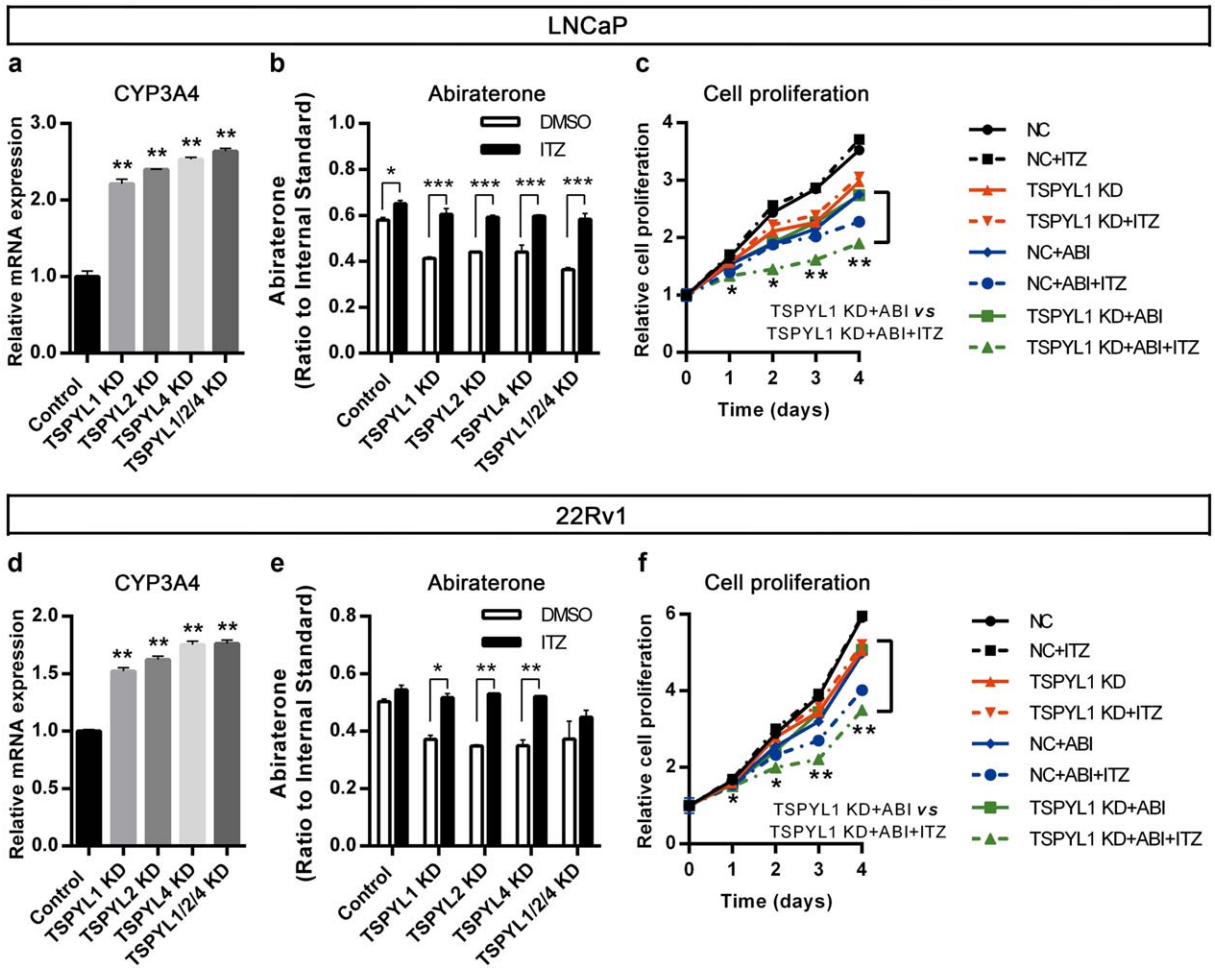

Figure 3 Changes of *CYP3A4* expression, abiraterone levels, and cell proliferation after the knockdown of *TSPYL1*, *TSPYL2*, and *TSPYL4* individually or all three together in prostate cancer cells treated with abiraterone alone or in combination of itraconazole. LNCaP and 22Rv1 cells were pretreated. mRNA expression of CYP3A4 was determined by qRT-PCR using ACTIN as an internal control. 10 nM of the CYP3A4 inhibitor itraconazole (ITZ) or its vehicle control, DMSO, was added in combination with 1 μM abiraterone and the level of abiraterone was then analyzed by high-performance liquid chromatography (HPLC). Anastrozole was added during the compound extraction step as an internal standard for the HPLC. Relative cell proliferation was determined by MTS assay. mRNA levels were compared in cells with specific TSPYL knockdown vs. cells transfected with control siRNAs in three independent experiments. Abiraterone levels were compared between cells treated with ITZ vs. no ITZ treatment. Cell proliferation was compared at each timepoint after various treatments. All statistical analyses were performed with the two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

nsSNPs showed differential effects on DNA-protein binding when compared to the WT (**Supplemental Figure S8d**).

CYP3A4 and CYP17A1 protein levels were also analyzed in NCI-H295R, LNCaP, and 22Rv1 cells overexpressing variant and WT expression constructs for TSPYL1 and TSPYL4. CYP2C9 and CYP2C19 were analyzed only in HepaRG cells because of low endogenous protein levels in the other cell lines. Again, the TSPYL1 P62S abolished TSPYL1's regulation of CYP3A4 levels in these cell lines (**Figure 4a,d**; **Supplemental Figure S6e**). None of the *TSPYL4* nsSNP affected the regulation of CYP3A4 and CYP17A1 levels (**Supplemental Figures S6f and S9**).

The rs3828743 SNP (P62S) abolished TSPYL1 regulation of the transcription of the abiraterone metabolizing enzyme, CYP3A4, but not CYP17A1, the target for abiraterone. That led us to test the effect of this SNP on the abiraterone level and response. WT and TSPYL1 P62S expression constructs were transfected into NCI-H295R, HepaRG, LNCaP, and 22Rv1 cells. As anticipated, overexpression of the variant construct showed no differential effect on DHEA level when compared with the WT, since the SNP did not affect CYP17A1 transcription (**Supplemental Figure S10**). We then measured abiraterone intracellular levels in the same four cell lines, overexpressing the WT and variant allozymes. Cell proliferation was also determined for the two prostate cancer cell lines. Overexpression of TSPYL1 WT protein, which downregulated CYP3A4 expression, resulted in a significant increase in abiraterone concentrations in all four cell lines tested (**Figure 4b,e**; **Supplemental Figures S6g and S8f**). Overexpressing WT *TSPYL1* also caused cells to be more sensitive to abiraterone (**Figure 4c,f**). However, overexpression of the TSPYL1 P62S reduced abiraterone levels and reduced sensitivity to abiraterone compared with cells overexpressing WT protein. We did not observe a further increase in the CYP3A4 level, abiraterone level, and cell proliferation in cells

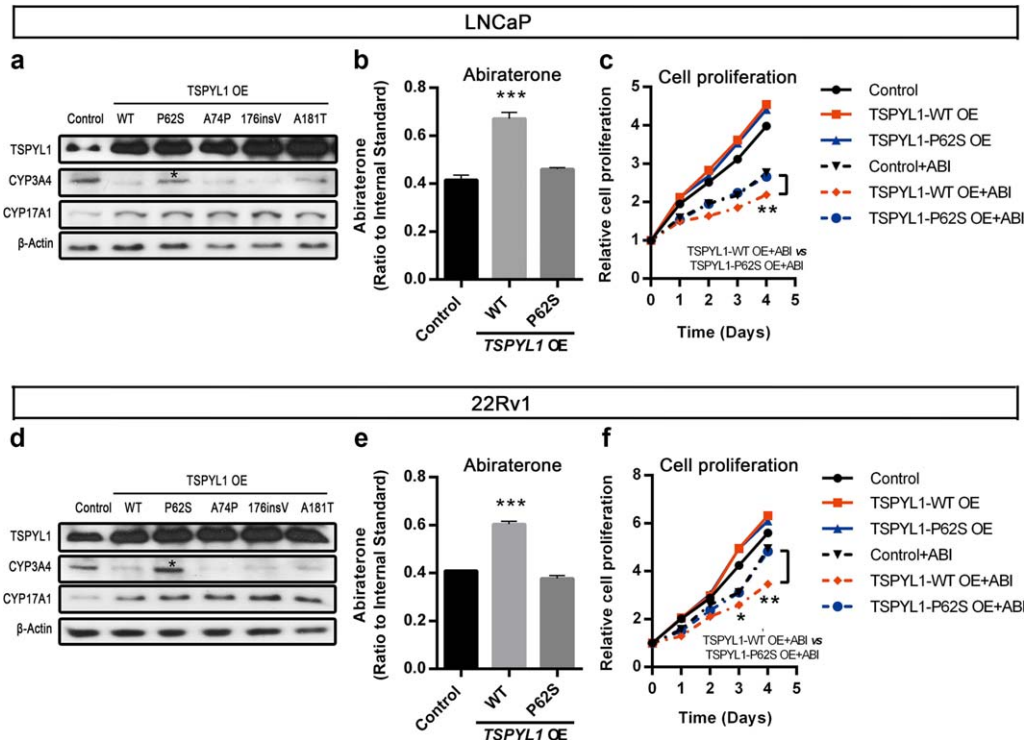

Figure 4 CYP3A4 and CYP17A1 protein levels (left panel), abiraterone levels (middle panel), and cell proliferation (right panel) were measured in prostate cancer cells overexpressing TSPYL1 WT or variant allozymes in the presence of various treatments as indicated. LNCaP and cells were pretreated as described in **Figure 3**. Abiraterone levels were compared between cells overexpressing WT or P62S allozymes and empty vector. Cell proliferation was compared between groups at each timepoint by the use of two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

overexpressing the P62S variant protein that were treated with abiraterone compared with vehicle, which probably was due to the high endogenous expression of CYP3A4 (**Figure 4b,e**; **Supplemental Figures S6g and S8f**). These results indicated that the *TSPYL1* P62S SNP might be a biomarker for abiraterone response in prostate cancer patients.

TSPYL1 rs3828743 nsSNP is associated with abiraterone acetate/prednisone response in CRPC patients

To determine whether the *TSPYL1* rs3828743 SNP was associated with abiraterone response in prostate cancer patients, we used data from a prospective clinical trial involving 89 patients with metastatic CRPC who had been treated with abiraterone acetate/prednisone (AA/P).[31] Of 89 patients recruited, 87 patients had information available for their initial response to AA/P. Initial response was defined by assessment of composite progression after 12 weeks of drug exposure that included concurrent evaluation of serum PSA, bone, and computed tomography (CT) imaging and symptom assessment with the Functional Assessment of Cancer Therapy-Prostate (FACT-P) scale. The patients' characteristics are shown in **Supplemental Table S4**. Fifty patients responded (no disease progression) to AA/P, while 37 patients did not **(Supplemental Table S5)**. **Supplemental Table S6** shows that the *TSPYL1* rs3828743 SNP variant allele (A) was more frequently presented in nonresponders ($P = 0.013$), with an odds ratio of 2.47 (1.23, 4.96). All six patients who were homozygous AA genotype fell exclusively into the nonresponder group ($P = 0.007$ compared to GG) (**Table 1**), in agreement with *in vitro* data (**Figure 4**). Patients were followed up with disease assessment until this article was drafting. At the time of analysis, 69/87 patients had progressed (median study follow-up was 26.5 months; interquartile range (IQR) was (19.1, 32.7)). The KM plot showed that the AA genotype was significantly associated with poor progression-free survival (PFS medians of 3.0 months (IQR: 2.8–3.4) for AA, 7.6

Table 1 Proportions for each abiraterone response group by *TSPYL1* rs3828743 SNP genotype

	AA ($N = 6$)		AG ($N = 32$)		GG ($N = 49$)	
rs3828743 genotype	Number	Percentage (95% CI)	Number	Percentage (95% CI)	Number	Percentage (95% CI)
Responder	0	0.0 (0.0,45.9)	18	56.3 (37.7,73.6)	32	65.3 (50.4,78.3)
Nonresponder	6	100.0 (54.1,100.0)	14	43.8 (26.4,62.3)	17	34.7 (21.7,49.6)
P- value[a]			0.007			

[a]Fisher's Exact Test: AA vs. (AG+GG). Pairwise Fisher's Exact Test: AA vs. AG: $P = 0.021$, AA vs. GG: $P = 0.003$; AG vs. GG: $P = 0.486$.

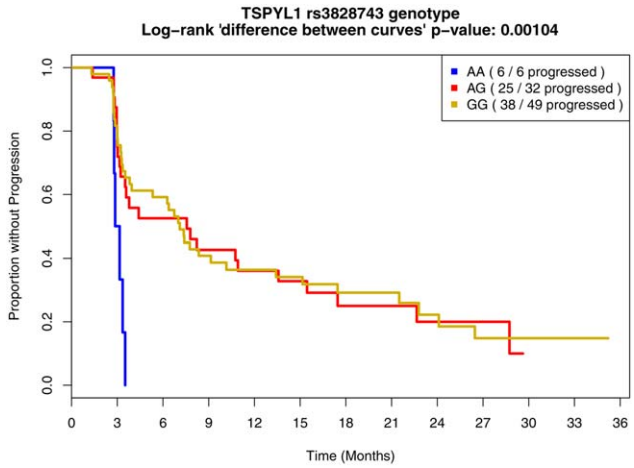

Figure 5 Kaplan–Meier survival curve showing the progression status of three groups of mCRPC patients with different genotypes AA ($N = 6$, blue), AG ($N = 33$, red), and GG ($N = 50$, orange). The *P*-value was calculated using the log-rank test.

months (IQR: 3.0–20.1) for AG, and 7.1 months (IQR: 3.2–22.8) for GG; log-rank test $P = 0.00104$) (**Figure 5**). A multivariate Cox regression analysis of rs3828743 genotype with PFS was also performed adjusting for potential predictive variables, including age ($< = 72$ vs. > 72), baseline PSA ($< = 10$ vs. > 10), Gleason score ($< = 7$ vs. 8-10), and metastatic volume (low vs. high). Patients with AA genotype significantly increased risk for having shorter PFS when compared to the GG genotype (hazard ratio (HR) = 3.36; 95% confidence interval (CI): 1.34–8.40; $P = 0.01$). Taken together, these results suggest that rs3828743 SNP might be a biomarker for AA/P response in mCRPC patients.

Since the *CYP17A1* rs2486758 SNP has been reported to be associated with the outcome of AA/P treatment in prostate cancer patients,[31] and since this *CYP17A1* SNP maps to the TSPYL1/2/4 binding sites within the promoter region (**Supplemental Figure 4d**), we also determined whether this SNP might affect TSPYL1/2/4 binding to the *CYP17A1* promoter. DNA fragments of *CYP17A1* promoter containing either WT or variant *CYP17A1* rs2486758 SNP genotypes were inserted into a luciferase reporter gene construct and luciferase activity was measured. We found decreased luciferase activity in cells with TSPYL1/2/4 knocked down and increased activity in cells with upregulation of TSPYL1/2/4 (**Supplemental Figure S11b,c**), consistent with the results of the ChIP assay (**Supplemental Figure S4**). However, the *CYP17A1* rs2486758 SNP variant did not affect the binding of either WT or the various TSPYL variant allozymes to the *CYP17A1*.

DISCUSSION

Inspired by our previous GWAS,[12] here we tested the hypothesis that TSPYL proteins might regulate the expression of multiple CYP genes. We found that TSPYL1, TSPYL2, and TSPYL4 are transcriptional regulators for CYP17A1, CYP3A4, CYP2C9, and CYP2C19. Specifically, these three TSPYLs induced CYP17A1 but suppressed CYP3A4 in hepatic, adrenal and prostatic cancer cells (**Figure 1**; **Supplemental Figures S1, S2, S3**). ChIP assays showed that TSPYL1, TSPYL2, and TSPYL4 could bind to the promoters of all four *CYPs* in the regions containing the same sequence motifs (**Supplemental Figure S4**). This regulation might also involve multiple proteins as described in a previous report.[32] Additionally, interactions among TSPYL1, TSPYL2, and TSPYL4 are predicted in the BioGRID.[33] These previous studies support the conclusion that TSPYL1, TSPYL2, and TSPYL4 might function in a transcriptional complex to regulate these CYPs. Future ChIP-sequencing and RNA-sequencing studies will be required to assess the role of TSPYLs in transcriptional regulation at a genome-wide level to help us understand the underlying biology of this family of proteins.

CYP3A4, CYP2C9, and CYP2C19 are important drug-metabolizing enzymes and their expression and activities show great interindividual variability. Genetic polymorphisms[34–36] and transcriptional regulation induced by drugs are known to result in interindividual variability in CYP activities. For instance, all three genes can be induced by rifampicin and St. John's wort through a nuclear receptor, the pregnane X receptor (PXR).[37–40] We have demonstrated that TSPYL1, TSPYL2, and TSPYL4 are transcriptional suppressors of the three drug-metabolizing CYPs, but inducers of CYP17A1, which might result from different coregulators recruited to each of the gene promoters. CYP17A1 belongs to the steroid-synthesizing CYP family. CYP17A1 transcription was promoted by activation of steroidogenic factor-1 (SF-1)[41,42] and suppressed by the farnesoid X receptor (FXR).[43] Our ChIP assay showed that TSPYL antibody-enriched DNA fragments mapped in a region $-266 \sim +151$ bp upstream from the *CYP17A1* transcriptional-start site (**Supplemental Figure S4d**). This region is known to contain two SF-1-binding elements.[41,42] SF-1, FXR, and PXR are all orphan nuclear receptors that share a similar protein structure.[44] It has been reported that a TSPYL1/TSPYL2-containing complex is a transcriptional coregulator of nuclear receptors such as NR4A1 and ERα.[32] Whether the TSPYLs interact with additional known CYP-regulating nuclear receptors needs to be further determined.

Most metastatic prostate tumors, even though they are castration-resistant, continue to grow.[45–47] Although a major source for intratumor androgens is circulating DHEA secreted from the adrenals, intratumor *de novo* androgens synthesized from cholesterol that are independent of circulating adrenal androgens may also contribute to CRPC progression.[27,28] Either way, decreasing the synthesis of DHEA by inhibiting CYP17A1 is clinically effective in the treatment of CRPC, and a novel inhibitor of CYP17A1, abiraterone, is FDA-approved to treat CRPC.[19] We observed that TSPYLs regulate both CYP17A1, the abiraterone therapeutic target, and CYP3A4, the major abiraterone-metabolizing enzyme. Those observations motivated us to determine whether TSPYLs can affect androgen synthesis and abiraterone metabolism in prostate cancer cells. Indeed, downregulation of all three *TSPYLs* decreased CYP17A1 expression and DHEA synthesis, not only in NCI-H295R adrenal cells, but also in two prostate cancer cells, LNCaP and 22Rv1. Most important, altering levels of the three TSPYLs had a significant impact on androgen synthesis and, thus, influenced prostate

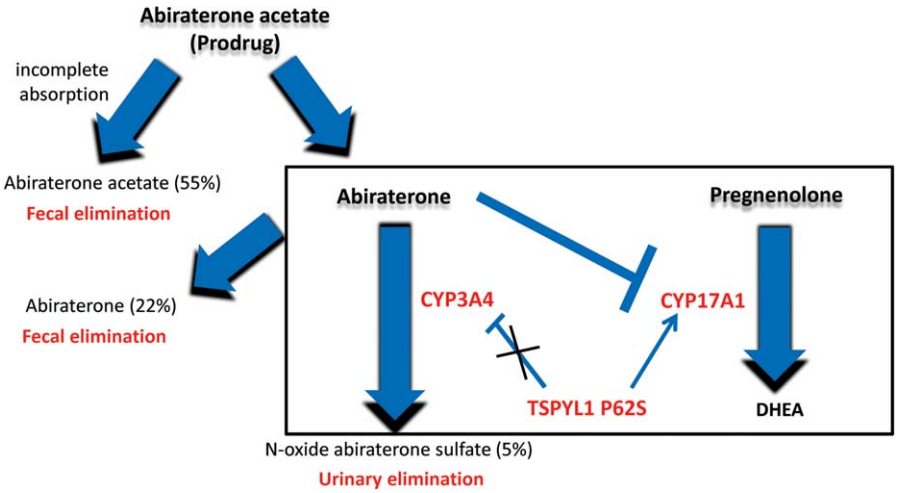

Figure 6 Schematic of postulated mechanism of the effect of *TSPYL1* rs3828732 SNP, from molecular pharmacology to efficacy outcome. Abiraterone acetate is administered via oral and around 55% of abiraterone and 22% abiraterone are recovered in the feces. Nearly 5% of abiraterone was eliminated via urine as N-oxide abiraterone sulfate. CYP3A4 is the major enzyme metabolizing abiraterone in liver and tumor. *TSPYL1* rs3828743 (P62S) abolishes TSPYL1's suppression on CYP3A4, resulting in increased CYP3A4 expression and decreased abiraterone exposure, which dramatically affects the inhibition of abiraterone on CYP17A1, and further, the drug response.

cancer cell proliferation (**Figure 2**; **Supplemental Figure S7**). In contrast to CYP17A1, CYP3A4 was upregulated after downregulation of individual TSPYLs in prostate cancer cells. CYP3A4 catalyzes the metabolism of the parent drug to form inactivate metabolites,[22] which also contributed to prostate cancer cell proliferation. The regulation of CYP3A4 and CYP17A1 by TSPYLs also raised the possibility of using specific CYP3A4 inhibitors to sensitize to abiraterone treatment. Finally, genetic variation in *TSPYLs* might have significant impact on interindividual variation in treatment response to abiraterone through the regulation of CYPs. After characterizing all the common variants in *TSPYLs* identified by the 1000 Genome Project, the *TSPYL1* rs3828743 SNP was found to affect TSPYL1-dependent transcriptional suppression of CYP3A4 but not its transcriptional induction of CYP17A, a phenomenon that might be due to different DNA structural environments in the promoter regions of these genes. Our ChIP assays showed that the TSPYL1 P62S allozyme showed decreased binding to the promoter region of *CYP3A4*. Indeed, the TSPYL1 P62S allozyme increased CYP3A4 expression and abiraterone metabolism when compared with the WT protein, which raised the possibility that patients with the *TSPYL1* rs3828743 SNP might be less responsive to abiraterone. This hypothesis was supported by both *in vitro* and patient data (**Figures 4**, **5**, **Table 1**).

Intriguingly, we also found that knockdown of TSPYLs inhibited proliferation in AR-positive triple negative breast cancer cells by decreasing the expression of CYP17A1 (data not shown), very similar to what we observed in prostate cancer cells, emphasizing the important role of TSPYLs in gene transcription regulation and treatment response.

Our model shown in **Figure 6** summarizes the effect of *TSPYL1* rs3828732 SNP on CYP3A4 and CYP17A1 through the regulation of the *TSPYL1* gene. The majority of abiraterone recovered in feces is the inactive prodrug, which may be due to incomplete absorption. Even though only 5% of abiraterone is eliminated mainly through the CYP3A4 metabolism pathway[48] (**Supplemental Figure S8e,f**), the intratumor *in situ* metabolism may be more important in determining abiraterone response than its systemic metabolism (**Figures 3**, **4**). Obviously, the patients in our study were also cotreated with prednisone, a substrate for CYP3A4, which could also contribute to the overall clinical outcomes that might be influenced by the *TSPYL1* SNP. Therefore, our clinical association results need to be further validated in future studies.

In summary, our findings suggest that genetic alterations in *TSPYLs,* together with known CYP polymorphisms, could have a significant impact on individual variation in response to therapy.

MATERIALS AND METHODS

Clinical trial consent and ethics

The AA/P clinical study (PROMOTE) (https://clinicaltrials.gov/identifier NCT #01953640) was reviewed and approved by the Mayo Clinic Institution Review Board (IRB), with written informed consent provided by all enrolled patients.

Clinical trial and data analysis

In all, 89 patients with mCRPC were enrolled in the study with two serial metastatic tissue biopsies collected at baseline and 12 weeks after abiraterone treatment. Exome sequencing was performed with tumor and germline DNA and the results were deposited in dbGAP (phs001141 (SRP082386)). The study and sequencing results are reported separately (Manish Kohli *et al.,* in review). To obtain the rs3828743 genotypes of the PROMOTE patients, we queried the exome-seq alignment files of the patient blood samples, using an in-house script.

Initial drug response was defined by assessment of composite progression after 12 week of drug exposure. In all, 87 patients had this information for analysis. The association of the *TSPYL1* rs3828743 SNP with abiraterone initial drug response was analyzed by Fisher's Exact Test.

PFS was based on the Prostate-Cancer Working Group-2 (PCWG2) definition, using the composite endpoint of the time from start trial

enrollment to the first PSA increase ≥25% and ≥2 ng/mL above the nadir (and subsequently confirmed by a second PSA value ≥3 weeks apart); or radiographic progression in soft tissue or bone. PFS was calculated from the time at enrollment to time of first progression event or censored at the time of the last evaluation. Survival analysis was performed using the R package "survival."[49] The *P*-value was calculated using the log-rank test.[50] Distributional statistics for follow-up time and progression-free survival time were obtained by Kaplan–Meier estimation. A multivariate Cox regression analysis investigating association of SNP (rs3828743) with PFS was performed adjusting for potential predictive variables, age (< = 72 vs. > 72), baseline PSA (< = 10 vs. > 10), Gleason score (< = 7 vs. 8-10), and metastatic volume (low vs. high).

Detailed descriptions of additional methods for all the experiments described in the article are included in the **Supplemental Materials and Methods**.

Additional Supporting Information may be found in the online version of this article.

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CONSENT AND ETHICS

The AA/P clinical study (https://clinicaltrials.gov/ identifier NCT # 01953640) was reviewed and approved by the Mayo Clinic Institution Review Board (IRB), with written informed consent provided by all enrolled patients.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

S.Q., D.L., and L.W.W. wrote the article; L.W.W., R.W., S.Q., D.L., and M.K. designed the research; S.Q., D.L., N.N., and J.Y. performed the research; L.W.G., P.V., and D.H. analyzed the data. The first two authors contributed equally to this work.

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