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14. ABSTRACT

The P582S C->T and A588T G->A polymorphisms in the Hypoxia-inducible factor-1α (HIF-1α) gene have been associated with enhanced stability of the protein and androgen-independent prostate cancer (CaP) During the course of our research we published that P582S polymorphism was not associated with CaP (see appendix). However we observed a significant interaction of the P582S genotype with insulin-like growth factor binding protein (IGFBP)-3 in modifying CaP risk such that higher IGFBP-3 levels (>= versus <median) were associated with a reduced risk only among men with the wildtype (OR, 95% CI = 0.74, 0.57-0.97; Pinteraction = 0.01). We therefore went on in the final year to study the effect of HIF 1 translation after treatment with agents that might down regulate IGF-1 down-stream signaling. Methods: Prostate cancer cell lines were treated with rapamycin, an mTOR antagonist, and the effect on HIF-1 protein levels was studied. Results: We found that agents such as rapamycin that might down regulate the effect of IGF-1 signaling by inhibiting the mTOR pathway, paradoxically increased HIF 1 protein levels. Conclusions : Treatment of prostate cancer with agents that attempt to affect signal transduction can have a paradoxical effect of HIF –1 protein levels by affecting its translation.

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None listed.

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Introduction:

The goals of this proposal were to assess the frequency of 2 specific polymorphisms in the hypoxia-inducible facltor 1 (HIF 1) alpha gene to assess the effect of these on the severity and incidence of prostate cancer. This was accomplished using a data set from the Men's Physician's Health Study here at Harvard. These data can be analyzed in the accompanying manuscript in the *Prostate*. This was the only aim that was approved in the study. Aim 2 was not funded. The work performed is best assessed in the accompanying manuscript included in the APPENDIX. , we found that, among men with homozygous wild-type CC, higher IGFBP-3 levels (>= versus <median) were associated with a 28% (95% CI, 0.55-0.95; $P_{interaction}$ = 0.01) lower risk of overall CaP and 53% (0.25-0.88; $P_{interaction}$ = 0.11) lower risk of metastatic/fatal CaP. The *A588T* polymorphism was too rare to assess interactions.
Conclusions: The two HIF-1α gene polymorphisms were not directly associated with CaP, but the interaction between the *P582S* polymorphism and IGFBP-3 merits further evaluation in mechanistic studies.

Our finding that linked HIF-1 alpha expression to the IGF-1 signaling axis lead to ongoing work in the final year of this proposal that is exhibited below. We sought to determine the effect of agents that inhibit the IGF-1 axis on protein levels of IGF-1. The work abouve demonstrated that HIF-1 levels that are higher as a result of a longer half life are moslt likely to be lead to more aggressive prostate cancer only in the setting of higher IGF-1 levels. What is the relationship between these two pathways if one exists? How might some of the newer agents being used for prostate cancer treatment affect HIF-1 protein levels?

Body: The goal of the this research proposal was to assess whether or not one of two specific polymorphisms in the HIF 1 alpha gene was associated with disease progression. Genetic variations in the gene encoding HIF-1α may also influence its function. Two germline single-nucleotide polymorphisms within the oxygen-dependent degradation (ODD)/pVHL binding domain (in exon 12) of the HIF-1α gene were identified (1). *P582S* causes a change from proline to serine at codon 582, and *A588T* causes a change from alanine to threoine at codon 588. Although the functionality of these polymorphisms are not completely clear, both the *P582S* (2) and *A588T* (2) variants (versus wild-type) yielded significantly higher transcription activity

We observed a statistically significant interaction between the HIF-1α P5282S polymorphism and baseline plasma IGFBP-3 levels in modifying prostate cancer risk. We have previously reported that circulating levels of IGF-I were positively (whereas levels of IGFBP-3 were inversely) associated with risk of advanced prostate cancer in the same study population (3). In this analysis, such associations of IGFBP-3 with prostate cancer disappeared among men carrying the variant T allele (*CT* or *TT*), whereas among those with the *CC* wild-type, high levels of IGFBP-3 were significantly associated with reduced the risk of overall prostate cancer, advanced-stage, metastatic and fatal prostate cancer. Feldser *et al.* (4) demonstrated that insulin, IGF-I, and IGF-2 induce expression of HIF-1α, which is required for expression of genes encoding IGF-2, IGFBP-2 and IGFBP-3. Recent studies have shown that IGF-I stimulates HIF-1α accumulation, HIF-1α nuclear translocation, and HIF-1 activity, resulting in increased VEGF expression (5). IGFBP-3 is a multifunctional protein that induces apoptosis utilizing both IGF-dependent and -independent mechanisms. Recent *in vivo* studies provided first evidence that IGFBP-3 has direct, IGF-independent inhibitory effects on angiogenesis. This may explain why we observed a significant interaction of the HIF-1α P582S polymorphism with IGFBP-3 but not with IGF-I. Our data suggest that the regulation of hypoxia-angiogenesis microenvironment might influence the effect of IGFBP-3 on prostate cancer development and angiogenesis

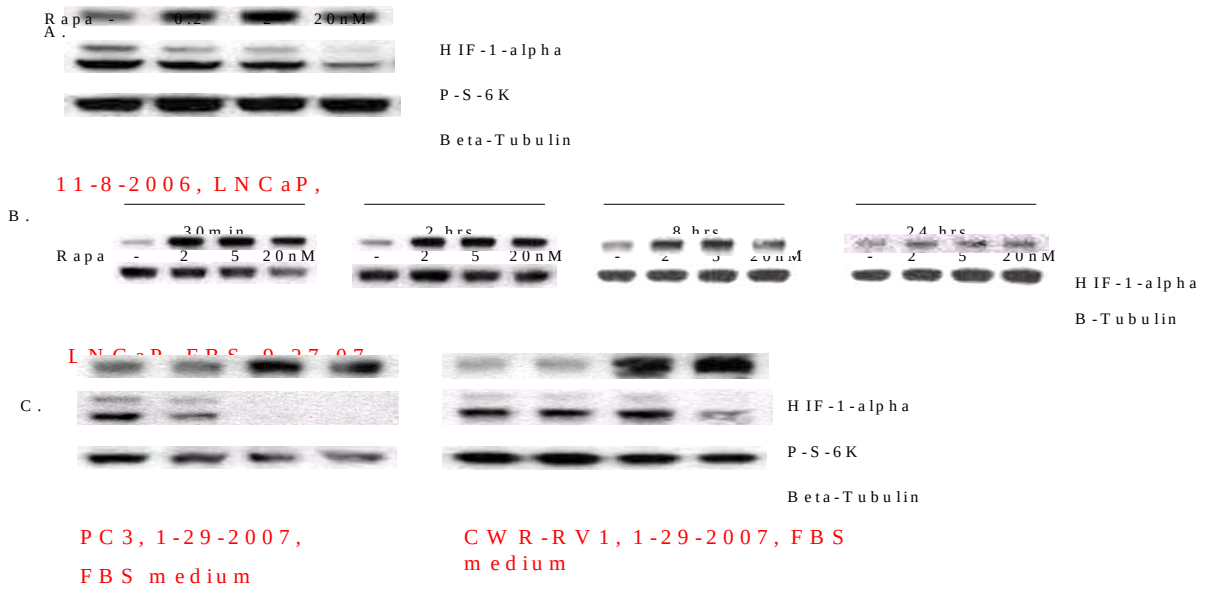

Figure 1. Low doses of Rapamycin stimulates HIF-1protein expression. This stimulation occurs rapidly and can last more than 24hrs. This effect is cell line and AR independent.

One of the surprising outcomes of our paper included in the APPENDIX below, is that there is an association between the IGF-1 signaling axis and HIF-1 alpha levels. Therefore samples from patients with a polymorphism associated with a longer half life of HIF-1 were only likely to have more aggressive disease if they had higher IGF-1 or IGFBP-3 levels. Although at the present time, there are no inhibitors of IGF-1 signaling directly, we can inhibit the pathway with down stream PI3kinase/akt/mTOR antagonists. When we used rapamycin to inhibit the mTOR pathway, we see a paradoxical increase in HIF-1 protein levels.

We sought to determine the mechanism behind this effect by exploring whether rapamycin altered translation of HIF-1 alpha. In the experiment shown below, we demonstrate that inhibition of mTOR leads to inhibition pf phosphorylation of phsopho-S6 (second row), but a paradoxical increase in activated elongation factor 4 (4th row).

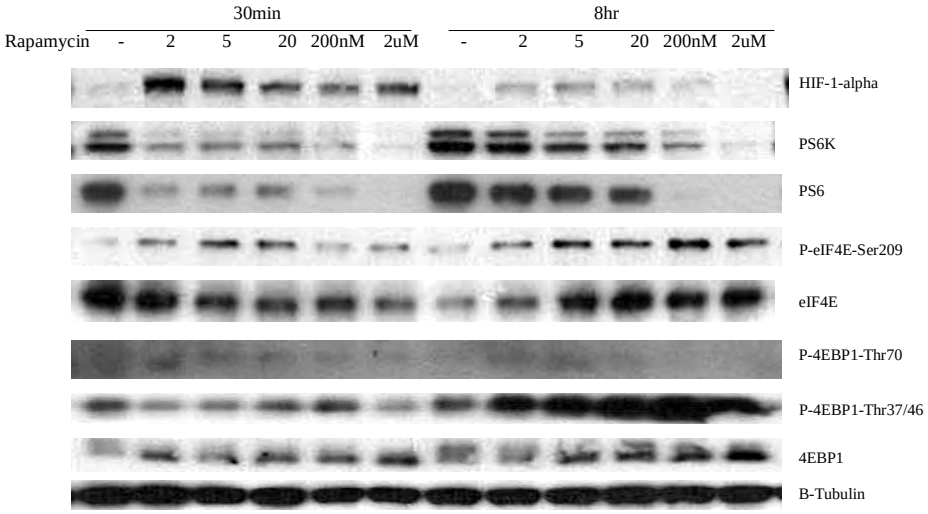

Figure 3. One potential mechanism of HIF-1 induction is by increased protein translation, in terms of stimulation of 4EBP1 and eIF4E phosphorylation.

Key Research Accomplishments:

1.) There is an interaction between a polymorphism in the HIF –1 alpha gene at site P582S and IGF-1 signaling resulting in more aggressive prostate cancer.
2.) Attempts at inhibiting IGF-1 signaling by inhibiting the mTOR pathway have a paradoxical affect on HIF-1 protein levels.

Reportable Outcomes

See enclosed manuscript by Li et al

Conclusions

References

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Appendix

Li et al; Bubley GJ; Balk SP, Gaziano JM; Pollak M, Stampfer MJ, Ma J. Hypoxia-inducible factor 1alpha gene polymorphisms, circulating insulin like growth factor binding prtoen-3 levels and prostate cancer; Prostate 67:1354-1351; 2007

The Prostate 67:1354–1361 (2007)

Hypoxia-Inducible Factor-1α (HIF-1α) Gene Polymorphisms, Circulating Insulin-Like Growth Factor Binding Protein (IGFBP)-3 Levels and Prostate Cancer

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BACKGROUND. The Hypoxia-inducible factor-1 (HIF-1) plays an important role in regulating angiogenesis in response to hypoxia. Two non-synonymous polymorphisms (*P582S C→T* and *A588T G→A*) in the coding region of the subunit 1α (HIF-1α) gene have been associated with enhanced stability of the protein and androgen-independent prostate cancer (CaP). Insulin-like growth factor binding protein (IGFBP)-3 mRNA is more abundantly expressed in hypoxia-related inflammatory angiogenesis and recent in vivo data suggest that IGFBP-3 has direct, IGF-independent inhibitory effects on angiogenesis.

METHODS. We examined the association of these polymorphisms with CaP among 1,072 incident cases and 1,271 controls, and further explored their joint associations with various prediagnostic plasma vascular endothelial growth factor (VEGF), IGF-I, and IGFBP-3 levels.

RESULTS. Neither the *P582S* nor the *A588T* polymorphism was associated with risk of overall or metastatic/fatal CaP. However, we found that, among men with the homozygous *CC* wild-type (but not *CT/TT*) of the HIF-1α *P582S*, higher IGFBP-3 levels (≥ vs. <median) were associated with a 28% (95% CI, 0.55–0.95; $P_{interaction} = 0.01$) lower risk of overall CaP and a 53% (0.25–0.88; $P_{interaction} = 0.11$) lower risk of metastatic and fatal CaP. The *A588T* polymorphism was too rare to assess interactions.

CONCLUSIONS. The two HIF-1α gene polymorphisms were not directly associated with CaP, but the interaction between the *P582S* polymorphism and IGFBP-3 merits further evaluation in mechanistic studies. *Prostate 67: 1354–1361, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: **insulin-like growth factor (IGF)-I; vascular endothelial growth factor (VEGF); prospective; nested case-control study**

INTRODUCTION

Hypoxia-inducible factor-1 (HIF-1), composed of an α and a β subunit [1], is a pivotal regulator of cellular response to hypoxia [2]. The transcriptional activity of HIF-1 is determined by the oxygen-regulated expression of the HIF-1α subunit [3], which is hydroxylated and degraded rapidly under normoxia through von Hippel–Lindau (VHL) mediated ubiquitination

Haojie Li and Glenn J. Bubley contributed equally to this work.

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whereas it becomes stabilized or even induced in response to hypoxia [3]. HIF-1α is overexpressed in many tumors [4,5] and a significant association between such overexpression and mortality has been reported for many cancer types [2]. In prostate cancer cell lines, expression of HIF-1α protein is positively associated with cell growth rates and metastatic potential [6,7]. In humans, expression levels of HIF-1α are upregulated in high-grade prostate intraepithelial neoplasia (PIN) lesions (vs. adjacent normal tissue) and are further enhanced in primary and metastatic prostate cancer [4,8,9].

Two germline single-nucleotide polymorphisms within the oxygen-dependent degradation (ODD)/pVHL binding domain (in exon 12) of the HIF-1α gene were identified [10]. *P582S* causes a change from proline to serine at codon 582, and *A588T* causes a change from alanine to threoine at codon 588. Although the functionality of these polymorphisms are not completely clear, both the *P582S* [11–13] and *A588T* [11] variants (vs. wild-type) yielded significantly higher transcription activity. Interestingly, our group recently identified the *P582S* C→T as a somatic mutation in bone marrow metastatic biopsies from men with androgen-independent prostate cancers; and our in vitro experiment demonstrated that, under normoxic conditions, this mutation had significantly higher transcription activity, which reflected increased HIF-1α protein expression [13]. Another group also found the same somatic mutation in prostate tumors [14]. One clinical case-control study found that the *P582S T*-containing variant genotypes were more common among men with androgen-independent prostate cancer [15]. Taken together, these data suggest that the HIF-1α polymorphisms might be related to risk of prostate cancer, especially the more aggressive form, and provide the rationale for the current investigation.

HIF-1 plays a major role in tumor progression and metastasis through activation of various genes that are linked to regulation of angiogenesis, cell survival, energy metabolism and apoptotic and proliferative responses [2,16]. Under hypoxic conditions, HIF-1 activates the vascular endothelial growth factor (VEGF) gene, leading to increased production of VEGF, a potent stimulator of angiogenesis [17,18]. Treatment of cultured cells with insulin, insulin-like growth factor-I (IGF-I), or IGF-2 induces HIF-1α protein expression, which is in turn required for expression of IGF-2 [19–22]. These activities may promote cell proliferation and survival. For these reasons, HIF-1 has become an attractive target for the development of anti-cancer drugs [2,23]. IGFBP-3 mRNA is more abundantly expressed in hypoxia-related inflammatory angiogenesis [24,25], perhaps as part of a feedback mechanism. In vitro and in vivo studies suggest that IGFBP-3 attenuates prostate tumor growth [26] and has direct IGF-independent inhibitory effects on angiogenesis [27], thus may inhibit tumor progression.

Using a nested case-control design within the Physicians' Health Study, we investigated the associations of the HIF-1α *P582S* and *A588T* polymorphisms with prostate cancer incidence and survival. We hypothesized that the HIF-1α *P582S* (*CC* or *CT*) and *A588T* (*GA*) variant genotype may be associated with increased risk, especially with aggressive disease, and poorer survival after diagnosis. We and other previously showed that circulating levels of IGF-I are positively (whereas levels of IGFBP-3 are mostly inversely) associated with risk of prostate cancer [28–30], especially advanced-stage disease [28,29], therefore we further explored the relationship between these HIF-1α polymorphisms and prediagnostic plasma levels of IGF-I, IGFBP-3, and VEGF in relation to prostate cancer incidence and aggressiveness.

MATERIALS AND METHODS

Study Population

The Physicians' Health Study (PHS) was a randomized, double-blind, placebo-controlled trial of aspirin and β-carotene among 22,071 healthy U.S. male physicians, aged 40–84 years, that began in 1982 [31]. During 1982 and 1984, 14,916 (68%) provided baseline blood samples prior to randomization [32], and more than 70% of the specimens were received between September and November in 1982. Through mailed baseline and follow-up questionnaires, we collected information on diet, lifestyle behaviors and medical history. Follow-up of the participants for morbidity and mortality is 97% complete to March 2005. Since 2003, information regarding disease progression and metastases was obtained from men with prostate cancer cases via additional follow-up questionnaires. Thus far, 87% of the eligible participants have responded, and less than 2% of participants have refused to participate or cannot because of cognitive problems.

Prostate cancer cases for the current study were drawn from participants who provided blood specimens at baseline and reported a diagnosis of prostate cancer between 1982 and 2000. Study investigators, unaware of the questionnaire or assay data, verified the reports of prostate cancer by participants and reviewed medical records and pathological reports to determine the tumor Gleason score, grade, and stage, according to the modified Whitmore-Jewett classification scheme [33]. The corresponding TNM staging to the Whitmore-Jewett system is as: stage A (T1N0M0), B (T2N0M0), C (T3N0M0), and D (TxN0M1, TxN1M0 or TxN1M1). We defined advanced-stage cancer as stage C and D diseases, and defined high-grade cancer as those with

Gleason score of 7 to 10 and those that were poorly differentiated. For each case, we selected one or two controls at random from those who had provided blood, were alive, had not had a prostatectomy, and had not reported a diagnosis of cancer (except for non-melanoma skin cancer) at the time the diagnosis was reported by the case subject. Controls were individually matched to cases by age (±1 year when feasible, else up to ±5 years for older men) and smoking status (never, former, or current).

Laboratory Assessment

The HIF-1α gene polymorphisms. DNA was extracted from baseline blood specimens for these men. With the laboratory personnel blinded to the case-control status, all samples were genotyped using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) at the Dana Farber/Harvard Cancer Center Genotyping Core. The HIF-1α-RFLP (*P582S* and *A588T*) genotypes were determined by the PCR amplification, followed by restriction enzyme digestion, as described previously [10]. The lab personnel were unaware of case-control status. To assess genotyping reproducibility, they repeated a random 10% selection of the samples; all genotypes matched initial designated genotypes.

Plasma concentrations of IGF-I, IGFBP-3, and VEGF. Samples from cases and their matched controls were assayed in the same batch to minimize interassay variability, and aliquots from a pool of quality control plasma (10% of total sample) were inserted randomly. Laboratory personnel were unable to distinguish among case, control, and quality control samples. Plasma levels of IGF-I and IGFBP-3 were assayed by enzyme-linked immunosorbent assay with reagents from Diagnostic Systems Laboratory (Webster, TX) in the laboratory of M. N. Pollak at the Lady Davis Research Institute of the Jewish General Hospital and McGill University. The median intra-assay coefficients of variation (CV) for IGF-I, and IGFBP-3 from the blinded quality control samples were 4.5% and 4.0%, respectively. Plasma concentrations of VEGF for each man were assayed in duplicate using a microplate luminescence detection system (Dynex Technologies, Chantilly, VA) and a human VEGF immunoassay Quantiglo kit (R&D Systems, Minneapolis, MN). The median CV for the quality control samples was 22.8%. Additional details of the VEGF assay are described elsewhere [34].

Statistical Analysis

We studied 1,072 men who developed prostate cancer during 18 years of follow-up and 1,271 controls. Of these, 1,041 cases (and 1,234 controls) were assayed for the HIF-1α *P582S* polymorphism and 1,066 cases (and 1,264 controls) for the HIF-1α *A588T* polymorphism. Because the majority (94%) of men were Caucasians and analyses with and without the non-Caucasian men yielded similar results, we included men of all ethnic groups in this study to maximize the sample size.

We compared allele and genotype frequencies between cases and controls with the χ^2-test. We examined the association of HIF-1α gene polymorphisms with risk of developing prostate cancer, then refitted models for men who developed metastases or died from prostate cancer during the follow-up, and within subgroups based on disease aggressiveness (by stage and grade), age at diagnosis (<65 years or ≥65 years), or PSA-era (before or after 1990). Because very few men (<2%) carried the HIF-1α *P582S TT* genotype, we use the dominant model by combining men with the *CT* or the *TT* genotypes into a single group and compared them with the reference group (the wild-type) for all analyses. The results were similar from both conditional (matched) and unconditional (unmatched) logistic regression analyses, so we primarily report estimates (relative risks, RR and 95% confidence intervals, 95% CI) from unconditional logistic regression, adjusted for matching factors of age, smoking status, and duration of follow-up. The unconditional logistic regression model permits all control subjects to be included in each model for maximizing statistical power, which is especially important for the subgroup analyses. To assess the influence of these polymorphisms on prostate cancer-specific survival, with survival time calculated from date of diagnosis to date of death from prostate cancer or other causes, or the end of the follow-up in March 2005, hazard ratios (HRs) and 95% CIs were estimated using Cox proportional hazards models, adjusting for age at diagnosis and smoking status.

The joint associations of HIF-1α polymorphisms with plasma levels (categorized by median) of IGF-I, IGFBP-3, and VEGF on prostate cancer were examined for the HIF-1α P582S genotype only. The frequency of HIF-1α *A588T* (*GA*) variant was too rare (<2%) to examine interactions. Conditional logistic regression models were implemented. Included in these analyses were 661 cases and 661 matched controls for IGF-I and IGFBP-3 and 397 cases and 398 matched controls for VEGF. Because levels of IGF-I and IGFBP-3 were correlated ($r = 0.46$, $P < 0.0001$) and these two biomarkers have opposite effects on risk, it was necessary to adjust for them simultaneously. We further compared the age-, smoking status-, and batch-adjusted levels of IGF-I and IGFBP-3 and age- and smoking status-adjusted VEGF levels with the presence or absence of

the HIF-1α *P582S* variant (*CT* or *TT*) genotype, using general linear regression models. All statistics were calculated using SAS (version 8.12; SAS Institute, Inc., Cary, NC) with a two-sided significance level of 0.05.

RESULTS

On average, men were 69 years of age when prostate cancer was diagnosed. The median interval from baseline in 1982 to diagnosis was 11.0 years for these cancer patients, and the average follow-up duration after diagnosis was 9.2 years. Among control subjects, the genotype frequencies were 80.6% (*CC*), 17.9% (*CT*) and 1.5% (*TT*) for the *P582S* polymorphism and 98.7% (*GG*), 1.3% (*GA*) for the *A588T* polymorphism (no instances for the *A588T AA* variant genotype). The genotype distributions were in Hardy–Weinberg equilibrium. The genotype frequencies were similar to previous reports for Caucasian populations [10,15]; however, compared with a report in a Japanese population [11], the *A588T* polymorphism in these men was rare (1.3% in our controls vs. 8.2% in Japanese controls). Genotype frequencies for the HIF-1α *P582S* and *A588T* polymorphisms did not differ significantly between cases and controls (Table I).

The *P582S* polymorphism was not associated with risk of overall prostate cancer (Table II, RR, 95% CI for *P582S CT* or *TT* vs. *CC* = 1.14, 0.93–1.40), regardless of age at diagnosis or calendar time (by PSA era), and was not related to prostate cancer-specific survival (HR, 95% CI for *P582S CT* or *TT* vs. *CC* = 0.90, 0.60–1.35). The HIF-1α *P582S* polymorphism had no relationship with risk for advanced-stage, metastatic and fatal disease. We also found no association of the *A588T* polymorphism with prostate cancer risk and survival; however, this polymorphism was too rare in this population to draw meaningful conclusions.

Plasma levels of IGF-I, IGFBP-3, and VEGF measured from blood samples collected at the baseline, an average of 11 years before cancer diagnosis, were not significantly different between cases and controls or by the HIF-1α *P582S* genotype (Table III). We found no interaction of the HIF-1α *P582S* polymorphism with baseline levels of VEGF or IGF-I. Men with the *CT* or *TT* genotypes and higher plasma IGF-I levels tended to have higher risk of overall, advanced-stage, high-grade, metastatic, and fatal prostate cancer, but none of these were statistically significant. However, we found that, among men with the *CC* (but not *CT*/*TT*) wild-type of the HIF-1α *P582S*, higher IGFBP-3 level

TABLE I. Baseline Characteristics of Prostate Cancer Cases and Controls in the Physicians' Health Study

	Cases (n = 1,072)	Controls (n = 1,271)
Age at baseline (year)[a]	58.9 ± 8.3	59.0 ± 8.1
Age at diagnosis (year)	69.4 ± 7.3	NA
Smoking status (%)[a]		
Current	9.5	8.9
Former	42.7	42.6
Disease aggressiveness, N (%)		
Stage AB	581 (54.2)	NA
CD	233 (21.7)	
Unknown	258 (24.1)	
Grade[b]		
Low	661 (61.7)	NA
High	384 (35.8)	
Unknown	27 (2.5)	
Metastatic/fatal prostate cancer[c]	184 (17.2)	NA
HIF-1α gene polymorphism, N (%)[d]		
P582S _CC	818 (78.6)	995 (80.6)
P582S _CT	209 (20.1)	221 (17.9)
P582S _TT	14 (1.3)	18 (1.5)
A588T_GG	1,053 (98.8)	1,247 (98.7)
A588T_GA	13 (1.2)	17 (1.3)

[a]Matching variable.
[b]High-grade cancer included Gleason 7–10 or poorly differentiated tumors.
[c]Metastatic/fatal prostate cancer, cases died of prostate cancer and those who developed metastasis during the follow-up.
[d]Data availability: HIF-1α *P582S* genotype, 1,041 cases and 1,234 controls; HIF-1α *A588T* genotype, 1,066 cases and 1,264 controls.

TABLE II. HIF-1α *P582S* Gene Polymorphism and Risk of Prostate Cancer[a]

	HIF-1α *P582S* Polymorphism			
	CC		*CT* or *TT*	
	N	RR (Ref.)	N	RR (95% CI)
Number of controls	995		239	
Overall prostate cancer	818	1.00	223	1.14 (0.93–1.40)
By tumor stage[c]				
Stage AB	436	1.00	129	1.23 (0.97–1.57)
Stage CD	178	1.00	49	1.14 (0.81–1.62)
By tumor grade[b,c]				
Low	506	1.00	133	1.10 (0.86–1.39)
High	288	1.00	88	1.28 (0.97–1.69)
Metastatic/fatal prostate cancer[d]	139	1.00	38	1.15 (0.77–1.70)
By age at diagnosis				
<65 years	227	1.00	65	1.10 (0.77–1.58)
≥65 years	591	1.00	158	1.14 (0.90–1.44)
By PSA era				
Pre-PSA (1982–1990)	232	1.00	55	0.92 (0.63–1.34)
Post-PSA (1991–2000)	586	1.00	168	1.21 (0.96–1.53)

[a]Risk ratio (RR) and 95% confidence interval (95% CI); unconditional logistic regression, adjusting for age at study onset and smoking status at baseline (never, past and current) and duration of follow-up.
[b]High grade was defined as Gleason 7–10 or poorly differentiated.
[c]Cases with unknown stage or Gleason sum were excluded from the analyses.
[d]Metastatic/fatal prostate cancer, cases died of prostate cancer and those who developed metastasis during the follow-up.

(≥ vs. <median) was associated with a reduced risk of overall prostate cancer (RR, 95% CI = 0.72, 0.55–0.95, $P_{interaction} = 0.01$; Table IV). The patterns were similar and the RRs were even lower for advanced-stage (RR, 95% CI = 0.45, 0.25–0.82) and for metastatic and fatal disease (RR, 95% CI = 0.47, 0.25–0.88); however, formal tests for interactions were not statistically significant, which can be due to small number of cases therefore limited power in these subgroup analyses.

DISCUSSION

In this population of primarily Caucasian U.S. physicians, we found no direct association between

TABLE III. Prediagnostic Plasma Levels of IGF-I, IGFBP-3 and VEGF by HIF-1α *P582S* Genotype[a]

	HIF-1α P582S CC		HIF-1α P582S CT or TT	
	N	Mean (SE)	N	Mean (SE)
IGF-I[a]				
Cases	577	192.6 (2.9)	155	203.1 (4.9)
Controls	557	191.2 (2.9)	130	191.1 (5.4)
IGFBP-3[b]				
Cases	578	3,145 (33)	155	3,261 (56)
Controls	561	3,187 (33)	130	3,081 (61)
VEGF[c]				
Cases	377	134.3 (34.4)	104	182.0 (58.8)
Controls	338	189.6 (36.5)	88	143.0 (63.8)

[a]Levels by genotype, $P > 0.05$.
[b]Age-, smoking status- and batch-adjusted levels.
[c]Age- and smoking status-adjusted levels.

TABLE IV. Joint Association of the HIF-1α *P582S* Polymorphism and Plasma Levels of IGF-I and IGFBP-3 With Prostate Cancer[a]

HIF-1α genotype	Plasma level[b]	IGF-I				IGFBP-3			
		No. of ca/co	RR	95% CI	$P_{interaction}$	No. of ca/co	RR	95% CI	$P_{interaction}$
Overall prostate cancer									
CC	Low	266/262	REF			282/251	REF		
	High	248/269	1.00	0.76–1.30		232/280	0.72	0.55–0.95	
CT or *TT*	Low	66/64	1.02	0.70–1.50	0.76	72/74	0.89	0.61–1.28	0.01
	High	77/62	1.34	0.90–2.00		71/52	1.20	0.78–1.84	
Advanced-stage prostate cancer[c]									
CC	Low	63/72	REF			68/54	REF		
	High	59/54	1.70	0.93–3.09		54/72	0.45	0.25–0.82	
CT or *TT*	Low	14/16	0.89	0.40–1.96	0.74	18/19	0.66	0.31–1.40	0.14
	High	19/13	2.01	0.87–4.64		15/10	0.92	0.32–2.62	
High-grade prostate cancer[d]									
CC	Low	94/96	REF			92/90	REF		
	High	72/89	0.83	0.52–1.33		74/95	0.81	0.51–1.28	
CT or *TT*	Low	25/16	1.66	0.82–3.37	0.79	31/21	1.53	0.81–2.90	0.74
	High	28/18	1.72	0.86–3.44		22/13	2.14	0.85–5.36	
Metastatic/fatal prostate cancer[e]									
CC	Low	59/52	REF			61/46	REF		
	High	41/55	0.80	0.43–1.49		39/61	0.47	0.25–0.88	
CT or *TT*	Low	12/12	0.84	0.36–1.98	0.48	18/15	0.95	0.43–2.10	0.11
	High	16/9	1.68	0.66–4.29		10/6	1.01	0.33–3.11	

[a]Conditional logistic regression, levels of IGF-I and IGFBP-3 were mutually adjusted in all models.
[b]Plasma levels of IGF-I or IGFBP-3, high and low were defined by wave-specific median among controls.
[c]Advanced-stage prostate cancer: stage C or D cancer at diagnosis; cases with unknown stage were excluded from the analyses.
[d]High grade was defined as Gleason 7–10 or poorly differentiated at diagnosis; cases with unknown grade were excluded from the analyses.
[e]Metastatic/fatal prostate cancer, cases died of prostate cancer and those who developed metastasis during the follow-up.

the HIF-1α *P582S* or *A588T* polymorphism and overall or metastatic/fatal prostate cancer. The *A588T* polymorphism was rare; only 13 cases and 17 controls (<2%) carried the *GA* variant genotype. The very low frequency of this polymorphism precludes definitive conclusions regarding its effect on prostate cancer incidence, aggressiveness or survivorship.

The lack of significant associations of the HIF-1α *P582S* polymorphism with aggressive prostate cancer in our study is somewhat at odds given previous observations. We detected a somatic *P582S* mutation in an androgen-independent prostate cancer case with metastasis to the bone [13] as did another group in prostate tumor [14]. A case-control study observed higher frequencies of the *P582S T*- containing variant genotypes among 196 androgen-independent prostate cancer cases compared with 196 controls [15]. Although proline 582 has not been identified as a hydroxylation site necessary to mediate VHL binding [35] and one study on colorectal cancer incidence reported null associations with this polymorphism [36], the *P582S* variant genotypes (vs. wild-type) yield significantly higher transcription activity under both normoxic and hypoxic conditions [11–13]. Furthermore, the *P582S* polymorphism may confer susceptibility to renal cell carcinoma [37,38] and is associated with significantly increased microvessels among patients with head and neck squamous cell carcinoma [11].

We observed a statistically significant interaction between the HIF-1α *P582S* polymorphism and baseline plasma IGFBP-3 levels in modifying overall prostate cancer risk ($P_{interaction} = 0.01$, Table IV) [28]. We have previously reported that circulating levels of IGF-I were positively associated (whereas levels of IGFBP-3 were inversely associated) with risk of advanced prostate cancer in the same study population [28]. In the current analysis, the associations of IGFBP-3 with prostate cancer was not observed among men carrying the variant T allele (*CT* or *TT*), whereas among those with the *CC* wild-type, high levels of IGFBP-3 were significantly associated with a 28% reduced risk of overall prostate cancer and over 50% reduced risks of high-stage, metastatic and fatal prostate cancer, although formal tests for interactions were not statistically significant for the subgroups. We recognize that these findings may just be a chance, but they are also

interesting given the fact that IGFBP-3 mRNA is more abundantly expressed in hypoxia-related inflammatory angiogenesis [24,25]; IGFBP-3 is a multifunctional protein that induces apoptosis utilizing both IGF-dependent and IGF-independent mechanisms [26]; and it has direct, IGF-independent inhibitory effects on angiogenesis in vivo [27]. Thus, larger studies are needed to confirm these exploratory findings and the interaction between the *P582S* polymorphism and IGFBP-3 merits further evaluation in mechanistic studies.

VEGF is a pleiotrophic growth factor that promotes endothelial cell proliferation, vascular permeability, and angiogenesis [39,40] and is overexpressed in prostate cancer [41]. Because it is shown that the binding of HIF-1α to the VEGF promoter is required for maximum transcription of VEGF mRNA following hypoxia [17,18], we had hypothesized that the HIF-1α *P582S* variant carriers may have higher circulating VEGF levels and therefore higher risk of prostate cancer. The large intra-assay variation of the VEGF measurement, the null association between plasma VEGF level and prostate cancer that we described previously [34] as well as the lack of the interaction with the *P582S* polymorphism in the current study might reflect the difficulty for precise measurements of VEGF in frozen plasma. Improving assays for circulating VEGF levels or its tissue expression would be worthwhile to further evaluate the potential role of VEGF in both carcinogenesis and angiogenesis.

Strengths of the current study include its prospective design, large sample size and complete long-term follow-up. Prospectively collected plasma enabled us to assess the levels of IGF-I, IGFBP3 and their interaction with the HIF-1α polymorphism. Although a single assessment of biomarker levels from the baseline blood is an imperfect indicator of long-term exposures, a pilot study with blood drawn from the same individuals at a 5-year interval showed a high correlation ($r = 0.75$) for IGF-1 (Ma, J, unpublished data). Furthermore, significant associations of plasma levels of IGF-I and IGFBP-3 with prostate cancer risk [28] also provide assurance that these biomarker data are valid in reflecting long-term status in our study population. One limitation of the current study is the limited statistical power for subgroup analyses. In testing for interactions, the low frequency of the *TT* variant genotype allows us to assess the dominant effect only, thus we may not exclude possible chance findings.

To summarize, we found no direct association between either the HIF-1α *P582S* or *A588T* polymorphism with prostate cancer. However, our data suggest a possible interaction between the *P582S* polymorphism and IGFBP-3 in modifying prostate cancer risk.

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