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PRINCIPAL INVESTIGATOR: Glenn J. Bubley, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

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14. **ABSTRACT**

Hypoxia-inducible factor-1 (HIF-1) plays an important role in tumor progression and metastasis and is overexpressed prostate cancer (CaP). Two polymorphisms (P582S C->T and A588T G->A) in the HIF-1α gene have been associated with enhanced stability of the protein and may confer susceptibility to androgen independent CaP. We examined the association of these two HIF-1α gene polymorphisms with CaP risk among 1,072 incident cases diagnosed during 18 years of follow-up and 1,322 age-matched controls in the Physician's Health Study We observed no association between the presence of these two polymorphisms and risk of total CaP. However, the HIF-1α P582S T variant allele carrier (CT or TT vs. CC) was associated with a nonsignificant increased risk of high-grade tumor (Gleason 7-10; OR, 95% CI =1.31, 0.97-1.75Among the T allele carriers, but not the CC wildtype carriers, men with higher IGF-I levels (>=median vs. <median) had increased risk for total (OR, 95% CI =1.55, 0.96-2.48), aggressive (stage C, D, Gleason 7-10, or fatal disease, OR, 95% CI = 2.15, 1.04-4.43), and fatal CaP (OR, 95% CI = 4.91, 1.27-18.9). Higher IGFBP-3 levels (>=median vs. <median) were associated with lower risk mainly among men with the homozygous CC genotype.

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I. Introduction

Hypoxia-inducible factor-1 (HIF-1) plays an important role in tumor progression and metastasis through activation of various factors that are linked to regulation of angiogenesis, cell proliferation and tumor growth. HIF-1α is overexpressed prostate cancer (CaP). Two polymorphisms (*P582S C->T* and *A588T G->A*) in the HIF-1α gene have been associated with enhanced stability of the protein and may confer susceptibility to androgen independent CaP.

We examined the association of these two HIF-1α gene polymorphisms with CaP risk among 1,072 incident cases diagnosed during 18 years of follow-up and 1,322 age-matched controls in the Physician's Health Study. We further explored the joint associations of these polymorphisms with prediagnostic plasma levels of insulin-like growth factor (IGF)-I, IGF binding protein (IGFBP)-3, and vascular endothelial growth factor (VEGF).

II. Body

Hypoxia-inducible factor-1 (HIF-1) is a pivotal regulator of cellular response to hypoxia (1). This functional protein, composed of an α and a β subunit (2), 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 (1, 3). HIF-1 has become an attractive target for the development of anti-cancer drugs (1, 4).

The transcriptional activity of HIF-1 is determined by the oxygen-regulated expression of the HIF-1α subunit (5). HIF-1α is hydroxylated and degraded rapidly

under normoxia through von Hippel-Lindau (VHL) mediated ubiquitination whereas stabilized or even induced in response to hypoxia (5). The hydroxylation site involves two critical proline residues, P402 and P564, and both of which are located in the oxygen-dependent degradation (ODD) domain (6, 7). HIF-1α is overexpressed in many tumors (8, 9) and significant association between overexpression and mortality has been reported for many cancer types (1). In prostate cancer cell lines, expression of HIF-1α protein is positively associated with cell growth rates and metastatic potential (10, 11). In human, expression levels of HIF-1α are up-regulated in high-grade prostate intraepithelial neoplasia (PIN) lesions (versus adjacent normal tissue) and are more enhanced in primary and metastatic prostate cancer (8, 12, 13).

In addition to the posttranscriptional, hypoxia-mediated regulation, the function of HIF-1α may also be influenced by genetic variation in the gene that codes for HIF-1α. Two single-nucleotide polymorphisms within the ODD/pVHL binding domain (in exon 12) of the HIF-1α gene were identified (14). *P582S* results in a change of an amino acid from proline to serine at codon 582, and *A588T* results in a change from alanine to threoine at codon 588. Although the functionality of these polymorphism are not completely clear, both the *P582S* (15-17) and *A588T* (15) variants (versus wild-type) revealed significantly higher transcription activity. For patients with head and neck squamous cell carcinoma, tumors with these two variants had significantly increased numbers of microvessels ($P = 0.02$) (15). These polymorphisms may also confer susceptibility to renal cell carcinoma (18, 19), androgen independent prostate cancer (20), and contributed to the progression or metastasis of these disease, although one study on colon cancer reported null associations (21).

With 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 and hypothesized that the HIF-1α *P582S* (*CC or CT*) and *A588T* (*GA*) variant genotype may be associated with increased risk, especially with aggressive disease. Based on the following evidence: 1) Treatment of cultured cells with insulin, insulin-like growth factor-I (IGF-I), or IGF-2 results in the induction of HIF-1α protein expression, which is in turn required for expression of mRNAs encoding IGF-2 and the IGF binding proteins (IGFBP-2 and IGFBP-3) cells (22-25); 2) circulating levels of IGF-I and IGFBP-3 predict risk of developing prostate cancer (26-28), especially advanced-stage disease (26, 27); and 3) production of vascular endothelial growth factor (VEGF), one of the most potent stimulators of angiogenesis, can be driven by hypoxia via transcription activation of the VEGF gene by HIF-1 (29, 30), 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 severity.

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). Men were excluded at baseline if they had a history of myocardial infarction, stroke, transient ischemic attack, or unstable angina; cancer (except for nonmelanoma skin cancer); current renal or liver disease, peptic ulcer, gout; or current use of platelet-active agents, vitamin A, or β-carotene supplements. The participants are predominately Caucasians (93%). 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 the

mailed-in baseline and follow-up questionnaires, we collected information on diet, lifestyle behaviors and medical history, and ascertain compliance and health endpoints. Follow-up of the participants for morbidity and mortality is 97% complete to March 2005. Since 2003, information, including disease progression and metastases, was obtained from alive PHS incident 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). 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, ±5 years for elderly participants) 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 (14). 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 1,062 men who developed prostate cancer during 18 years of follow-up and 1,297 matched controls. Of these, 1,031 cases (and 1,257 controls) had the HIF-1α *P582S* polymorphism and 1,056 cases (and 1,290 controls) had the HIF-1α *A588T* polymorphism. Because the majority (94%) of men were Caucasians and analyses with and without the non-Caucasian men provided the same conclusions, 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 total prostate cancer, then refitted models for men developed metastasis or died from prostate cancer during the follow-up, and within subgroups based on severity of disease (by stage and grade), age at diagnosis (< 65 years or >= 65 years), or PSA-era (before or after 1990). Advanced-stage cancer was defined as stage C and D diseases; and high-grade cancer included Gleason score of 7 and above and poorly-differentiated tumors. We performed both conditional (matched) and unconditional (unmatched) logistic regression analyses to estimate the relative risks (ORs) and 95% confidence intervals (CIs), using the wild-type group as the reference. Because the results were similar, we primarily reported results from unconditional logistic regression, adjusted for age at baseline, smoking status, and duration of follow-up, in consideration of the case-control selection criteria and matching. The unconditional logistic regression model permits all control subjects to be included in each model, especially those in the subgroup analyses, in order to maximize statistical power. The duration of follow-up was

calculated in years between baseline and diagnosis for cases, and the duration for a control was the same as the matched case.

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 very rare (< 2%), which did not allow for examination of the gene-hormone interactions. Since the frequency of the HIF-1α P582S *TT* genotype was also low (< 2%), we combined the *CT* and *TT* genotypes as one group and compared them with the reference group. Conditional logistic regression models were implemented. Included in this analyses were 684 cases and 684 matched controls for the models with IGF-I and IGFBP-3 and 419 cases and 425 matched controls for VEGF. Because levels of IGF-I and IGFBP-3 were correlated (n = 744, Pearson correlation coefficient r = 0.46, P<0.0001) and these two biomarkers may have opposite effects on risk, it was necessary to adjust for these two factors 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 by using SAS (version 8.12; SAS institute Inc, Cary, NC) with a two-sided significance level of 0.05.

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 80.7% (*CC*), 17.9% (*CT*) and 1.4% (*TT*)

for the *P582S* polymorphism and 98.5% (*GG*), 1.5% (*GA*) for the *A588T* polymorphism (no instances for the *A588T AA* variant genotype); and the genotype distributions were in accordance with Hardy-Weinberg equilibrium. The genotype frequencies were similar to previous reports for Caucasian populations (14, 20); however, compared with a report in a Japanese population (15), the *A588T* polymorphism in these men was rare. Cases did not differ significantly from controls for the characteristics presented (Table 1), including the genotype frequencies for the HIF-1α *P582S* and *A588T* polymorphisms.

For first goal of statement of work (polymorphisms and risk of disease acquisition): We observed no association between the presence of either of these two polymorphisms and risk of total prostate cancer, and the associations were similar by age at diagnosis or by PSA era (Table 2). The sample sizes were too small to perform subgroup analyses for the rare *A588T* polymorphism.

For second goal of statement of work (polymorphisms and disease severity)

Although it is unlikely the HIF-1α *P582S* polymorphism would predict advanced-stage, mestastatic and fatal disease, the presence of the *CT* or *TT* alleles *(vs. CC*) was associated with a nonsignificant increased risk of high-grade disease (RR, 95% CI =1.29, 0.98-1.70).

Plasma levels of IGF-I, IGFBP-3, and VEGF (Table 3) were not significantly different between cases and controls or by the HIF-1α *P582S* genotype. The HIF-1α *P582S* polymorphism may modify the associations of IGF-I and IGFBP-3 (but not VEGF) with prostate cancer. Compared with men with the wild-type (*CC*) and with baseline IGF-I levels below the median, the *T* allele carriers who had higher IGF-I levels

had the highest risk of total prostate cancer (RR=1.39, 95% CI = 0.94-2.06), and the risk increased more than 2-fold for advanced-stage tumor at diagnosis and metastatic and fatal disease. On the other hand, higher IGFBP-3 level (>= versus <median) was associated with a reduced risk of total prostate cancer (RR = 0.76, 95% CI = 0.58-0.99) primarily among men with the homozygous *CC* genotype ($P_{interaction}$ = 0.01), and the patterns were the same and the risk reduction was greater than 50% for advanced-stage and metastatic and fatal disease.

Key research accomplishments

1.) **Neither polymorphism in germ line DNA is a risk for prostate cancer acquisition. However, men with the wild type homozygous CC genotype and low IGF-BP3 levels had reduced chance of diease.**

2.) **The P582S polymorphism in men with high IGF-1 levels is associated with more aggressive cancers. Men with the wild type homozygous CC genotype and low IGF-BP3 levels had a greater than 50% decrease in having aggressive cancers**

Reportable outcomes: This work has resulted in a AACR abstract accepted for the 2006 meeting

Conclusions

In this population of U.S. physicians who are primarily Caucasians, we found no overall association between the HIF-1α *P582S* polymorphism and prostate cancer. However, men with the HIF-1α *P582S CT* or *TT* (*vs. CC*) genotypes had a nonsignificant increased (29%) risk of high-grade tumors (Table 2), and this polymorphism might interact with the IGF axis. We consistently observed that men with the *CT* or *TT* genotypes and high plasma IGF-I levels had the greatest risk of total, advanced-stage, metastatic and fatal prostate cancer; and high levels of IGFBP-3 seemed to be protective only among those with the *CC* wild-type, especially for advanced-stage, metastatic and fatal disease. The *A588T* polymorphism was too rare that only 13 cases and 19 controls (less than 2%) carried the *GA* variant genotype in this population, thus, the results were less conclusive

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Appendix:

Table 1. Baseline characteristics of prostate cancer cases and controls in the Physicians' Health Study

	Cases (n = 1072)	Controls (n = 1322)
Age at baseline (yr) [a]	58.9 ± 8.3	59.1 ± 8.1
Age at diagnosis (yr)	69.3 ± 7.3	NA
Smoking status (%) [a]		
Current	9.5	8.8
Former	42.6	42.7
Severity of disease, N (%)		
Stage AB	582 (54.3)	
CD	233 (21.7)	NA
Unknown	257 (24.0)	
Grade [b]		
Low	661 (61.7)	
High	384 (35.8)	NA
Unknown	27 (2.5)	
Metastatic/Fatal prostate cancer [c]	177 (16.5)	NA
HIF-1α gene polymorphism, N (%) [d]		
P582S _CC	818 (78.6)	1034 (80.7)
P582S _CT	209 (20.1)	229 (17.9)
P582S _TT	14 (1.3)	18 (1.4)
A588T_GG	1053 (98.8)	1295 (98.5)
A588T_GA	13 (1.2)	20 (1.5)

[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, 1041 cases and 1281 controls; HIF-1α *A588T* genotype, 1066 cases and 1315 controls.

Table 2. HIF-1α gene polymorphisms (*P582S* and *A588T*) and prostate cancer risk [a]

	HIF-1α *P582S* polymorphism				HIF-1α *A588T* polymorphism			
	CC		*CT or TT*		*GG*		**GA**	
	# case	OR (Ref.)	# case	OR (95% CI)	# case	OR (Ref.)	# case	OR (95% CI)
Number of controls	1034		247		1295		20	
Total prostate cancer	818	1.00	223	1.14 (0.93-1.40)	1053	1.00	13	0.75 (0.37-1.52)
By tumor stage [c]								
Stage AB	437	1.00	129	1.24 (0.97-1.57)	572	1.00	7	0.74 (0.31-1.76)
Stage CD	178	1.00	49	1.15 (0.81-1.63)	228	1.00	3	0.99 (0.29-3.42)
By tumor grade [bc]								
Low	506	1.00	133	1.10 (0.87-1.40)	649	1.00	8	0.75 (0.33-1.72)
High	288	1.00	88	1.29 (0.98-1.70)	377	1.00	5	0.78 (0.29-2.11)
Metastatic/Fatal prostate cancer [d]	133	1.00	37	1.17 (0.79-1.74)	172	1.00	1	0.48 (0.06-3.64)
By age at diagnosis								
<65 yrs	228	1.00	65	1.12 (0.79-1.61)	295	1.00	5	1.17 (0.37-3.64)
>= 65 yrs	590	1.00	158	1.14 (0.90-1.44)	758	1.00	8	0.60 (0.25-1.45)
By PSA era								
Pre-PSA (1982-1990)	232	1.00	55	0.92 (0.64-1.33)	288	1.00	3	1.38 (0.33-5.74)
Post-PSA (1991-2000)	586	1.00	168	1.21 (0.96-1.53)	765	1.00	10	0.59 (0.27-1.31)

[a] Unconditional logistic regression, adjusting for age at study onset and smoking status at baseline (never, past and current) and duration of follow-up; data availability: HIF-1α *P582S* genotype, 1041 cases and 1281 controls; HIF-1α *A588T* genotype, 1066 cases and 1315 controls.

[b] High grade was defined as Gleason 7-10 or poorly differentiated.

Table 3. Prediagnostic plasma hormone levels by HIF-1α *P582S* genotype

	HIF-1α *P582S CC*		HIF-1α *P582S CT or TT*	
	N	Mean (SE)	N	Mean (SE)
IGF-I[a]				
Cases	578	186.5 (2.4)	155	197.1 (4.7)
Controls	574	185.1 (2.4)	132	184.9 (5.1)
IGFBP-3[a]				
Cases	579	3166 (28)	155	3282 (53)
Controls	574	3214 (28)	132	3091 (58)
VEGF [b]				
Cases	377	134.5 (29.2)	104	182.3 (55.5)
Controls	351	184.2 (30.2)	90	151.3 (59.8)

[a] Age-, smoking status- and batch-adjusted levels.

[b] Age- and smoking status-adjusted levels.