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Polymorphism in endostatin, an angiogenesis inhibitor, and prostate cancer risk and survival: a prospective study

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Abstract

Endostatin inhibits endothelial cell proliferation and migration, prerequisites of angiogenesis. A functional missense mutation (*D104N*) in endostatin was associated with an increased prostate cancer risk in a small study. We undertook a larger, prospective study within the Physicians' Health Study to examine *D104N* and prostate cancer risk and progression among 544 incident prostate cancer cases (1982-1995) and 678 matched controls. The association between endostatin genotype and cancer risk was estimated using logistic regression models. Among cases, Cox models were used to assess *D104N* and lethal prostate cancer. Given the role of endostatin in neovascularization of adipose tissue, we cross-classified individuals on *D104N* genotype and body mass index (BMI). The genotype frequency was 1.3% homozygous (NN), 14.5% heterozygous (DN), and 84.2% wildtype homozygous (DD). There was no overall association between carriage of the N allele and prostate cancer risk (RR=1.2, 95% CI: 0.9-1.6) or cancer-specific mortality (HR=1.2, 0.7-1.8). Cases with the polymorphic allele were less likely to be overweight (BMI 25 kg/m^2 or greater, 26%) compared to men wildtype homozygous (48%), p<0.0001. Being overweight was associated with a 60% greater prostate cancer risk among those who were wildtype homozygous. In contrast, being overweight was associated a 50% lower risk of cancer among those with the N allele. We did not confirm earlier observation between the *D104N* polymorphism and prostate cancer. However, our data indicate that prostate cancer cases who carry the variant N allele are more likely to be overweight, and may be more susceptible to the angiogenic influences of obesity in prostate cancer pathogenesis.

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Keywords

Endostatin; angiogenesis; obesity; prostate cancer

Introduction

Angiogenesis is a discrete event in carcinogenesis that is related to the aggressive potential of a tumor.(1,2) Through the building of new vasculature, angiogenesis provides an independent blood supply essential for tumor growth and expansion.(3) Prostate cancer has the ability to produce angiogenenic factors (4), and there is accumulating evidence that increased angiogenenic properties within the tumor are associated with poor prostate cancer outcomes. (5,6)

Endostatin, a cleavage product of the C-terminal of collagen, is a potent inhibitor of endothelial cell proliferation and migration(4), requisites of angiogenesis. Endostatin acts exclusively on the endothelium, and induces endothelial cell apoptosis *in vitro*.(7) In animal models, systemic administration of endostatin increases apoptotic activity in tumor cells, decreases microvessel density, and regresses primary tumors.(8)

A missense mutation in the coding region for endostatin involves an asp1437-to-asn change in the *COL18A1* gene, a variant located at residue 104 in endostatin (*D104N*, rs1248337). The polymorphism does not appear to affect expression, since serum endostatin levels were similar among carriers and noncarriers of the variant allele.(9) Structural modeling analysis suggests that the polymorphism may affect protein conformation, which may subsequently impair the ability of endostatin to bind to other molecules.(9) While in cellular assays the variant allele did not influence binding to heparin, nor affect the ability of endostatin to inhibit endothelial cell migration, it did impair binding of endostatin to laminin.(10)

In a hospital-based, case-control study, Iughetti *et al* first examined endostatin genotype and prostate cancer risk, reporting a more than 2-fold increased risk among men who carried the variant allele.(9) In the present investigation, we present data from a larger, prospective study within the Physicians' Health Study examining the *D104N* endostatin polymorphism in relation to prostate cancer risk and survival. In addition to its role as an angiogenic inhibitor, endostatin influences neovascularization of nonneoplastic tissue such as adipose. (12) Accumulating data suggests a relation between body mass index (BMI) and prostate cancer progression.(13,14) Thus, we examined potential effect modification of BMI on prostate cancer risk and survival by the endostatin polymorphism.

Material and Methods

Study population

Participants in the present investigation come from the Physicians' Health Study, a randomized trial of aspirin and beta-carotene supplementation initiated in 1982 among 22,071 US male physicians ages 40-84. All were initially free of cancer at diagnosis. During 1982-1984, physicians were sent blood collection kits to obtain baseline samples prior to randomization. Prospectively collected samples are available from 14,916 (68%) of physicians, representing the study base for the nested case-control study.

Disease ascertainment

Participants are followed through annual questionnaires to collect data on lifestyle and medical factors, and biennally through postcards to ascertain compliance and health

endpoints. Follow-up of the participants for morbidity and mortality is 99% complete and ongoing (11).

Diagnosis of incident prostate cancer was self-reported by the participants and confirmed by study investigators through review of medical records and pathology reports. Information on tumor stage and grade was abstracted through these records. Prostate cancer death is confirmed through death certificate review, and information on post-diagnostic metastases collected through annual follow-up questionnaires of the men with prostate cancer.

We sought to select controls randomly from participants who had not had a partial or total prostatectomy or prostate cancer by the date of the case's diagnosis, and whom had sufficient plasma for biochemical analyses. Controls were matched, 1:1 or 1:2, to cases on age (±1 year, ±5 years for elderly participants) and smoking status (current, former, never). Given the low proportion (7%) of non-Caucasian males in the study, we limited this analysis to Caucasians in order to reduce possible bias from population stratification.

Included in this analysis were 544 incident prostate cancer cases diagnosed through 1995 and 678 controls with available genotyping data. Of the 544 cases, 147 (28.7%) were advanced stage tumors at diagnosis (Stage T3/T4/N1/M1), 223 (48.8%) had a high-grade tumor (Gleason 7 or higher, or poorly differentiated), and 129 (23.7%) of these cases developed lethal prostate cancer, defined as distant metastases or died of prostate cancer during follow-up. The mean age of cases and controls at baseline was 61 years (Table 1).

Genotyping of endostatin *D104N* polymorphism

Genomic DNA was prepared from frozen peripheral blood using "QIAamp DNA Blood mini kit" (QIAGEN Inc, Valencia, CA), quantitated by OD_{260}, and stored at 4°C. Genotypes were analyzed following to a previously established procedure(9) with modifications. Genomic DNA (200 ng) was amplified using 40 pmoles each of primers (Forward: 5-CACGGTTTCTCTTCCAGGAC-3′ and Reverse: 5′CTCTCAGAGCTGCTCACACG-3′) in 45 ul 1× Pfx amplification buffer (Invitrogen Corp., Carlsbad, CA) containing 0.56 mM $MgSO_4$, 0.33 mM dNTP, and 1 unit of Platinum Pfx DNA Polymerase (Invitrogen). Thirtytwo cycles of primary PCR amplification [denaturing (94°C, 40 sec), annealing (62°C, 30 sec), and extension (72°C, 30sec)] were performed. One ul product from a primary PCR was subjected to a secondary PCR using exactly the same condition as for the primary PCR. The reaction was maintained at 4°C after cycling. These PCRs yielded a 168 bp amplicon. Ten ul of the final PCR product was digested by incubating with 4 units of MseI (New England BioLabs, MA) at 37°C for 1 hr. Digested products were visualized on a 2% agarose gel stained with ethidium bromide. Fragment patterns specific for three genotypes were: D(Asp)/D(Asp) (GAC/GAC; 168 bp), D(Asp)/N(Asn) (GAC/AAC; 168, 101, 67 bp), and N(Asn)/N(Asn) (AAC/AAC; 101, 67 bp). To confirm genotypes, eighty-eight PCR products were randomly picked up and subjected to DNA sequencing using the same forward/reverse primers described above. Results showed identical genotypes for all these samples.

Statistical analysis

Unconditional logistic regression models were employed to examine the relation between endostatin and prostate cancer risk, controlling for matching factors. Given the low prevalence of men homozygous for the variant (1%), we combined men who carried at least one of the N alleles. Odds ratios, as an estimate of the relative risk, and 95% confidence intervals (95 % CI) were calculated, with men homozygous wildtype (DD) as the reference category. Given the role of angiogenesis in tumor aggressiveness and growth, we examined the association of the *D104N* polymorphism by prostate cancer stage and tumor grade. Moreover, in a survival analysis, among the prostate cancer cases, we explored whether men

who carried the N allele had a higher risk of prostate cancer death or developing metastases using proportional hazard regression models to account for potential confounding by age at diagnosis, tumor grade, and stage. Follow-up time was calculated from date of cancer diagnosis to development of distant metastases, prostate cancer death or censored at time of death from other causes or end of follow-up (March 1, 2008).

Results

Among controls, 84.2% men were wildtype homozygous (DD), 14.5% were heterozygous (DN), and 1.3% were homozygous carriers of the rare allele (NN) (Table 1) (permutation test of HWE: $p = 0.05$). The frequency of the N allele among the controls was similar to that previously reported.(15)

Carriage of the polymorphic N allele was not associated with risk of total prostate cancer risk (RR, 95% CI; 1.2, 0.9-1.6) (Table 2). The relative risks were similar for localized (1.3, 0.9-1.87) and advanced stage tumors (0.8, 0.5-1.4) at diagnosis, and did not differ according to tumor grade. Cancers diagnosed at an earlier age may indicate a familial component. In these data, however, there was no evidence that the endostatin *D104N* polymorphism was associated with prostate cancers diagnosed at a younger or older age (data available on request).

We undertook a survival analysis to explore the influence of the *D104N* polymorphism on risk of prostate cancer death or development of distant metastases over time (Table 2). Among the 544 incident cases, 129 men died of their cancer or developed metastases during follow-up through March 1, 2008. Controlling for clinical parameters, we found no association between carriage of the N allele and development of lethal prostate cancer (HR: 1.2, 95% CI: 0.8-1.8).

Interestingly prostate cancer cases with the polymorphic allele were less likely to be overweight or obese (26.0%) compared to men with the DD genotype (47.8%, $p<0.0001$). The mean BMI among cases was 24.9 kg/m^2 among those who carried an N allele versus 24.1 kg/m^2 for those who did not. There was no significant difference in the prevalence of overweight/obesity according to *D104N* genotype among the controls ($p\sim0.41$). Our group has previously shown within the PHS cohort that obese men have worse cancer survival compared to men with normal weight at baseline.(16) We therefore cross-classified men on BMI and *D104N* genotype for cancer risk and survival (Table 3). Among men homozygous wildtype for endostatin, we confirm the relation, demonstrating a 2-fold increase risk of prostate cancer among obese men (case control analysis), and a 2-fold increased risk of prostate cancer death or metastases over time among cases who were obese compared to those of normal weight (survival analysis). In contrast, among men who carried the polymorphic N allele, the relation with body mass index was inverse, such that the men who were healthy weight had a higher prostate cancer risk and a worse cancer prognosis.

Discussion

In this large, prospective study nested within the Physicians' Health Study, we observed no overall association between the *D104N* endostatin polymorphism and prostate cancer risk or prognosis. Our data show a consistently null association of the genotype for high grade or advanced stage tumors. Given its characterization as an angiogenesis inhibitor, one would hypothesis a stronger effect of the *D104N* endostatin polymorphism on advanced stage tumors or cancer progression if there were truly a relation.

Neovascularization of tumors requires the coordination of multiple pathways involving endothelial cells, extracellular matrices and soluble factors.(17,18) Each step is regulated by

angiogenesis inhibitors and inducers, and endostatin is one such inhibitor. Endostatin acts directly on endothelial cells to inhibit response to angiogenic signals, and accumulating evidence suggests a role for endostatin in inhibiting and reversing angiogenesis in prostate tumors. Transgenic mice predisposed to develop prostate cancer experienced greater survival when treated with endostatin compared to control. However, in a *Col18A1* knockout model, there was no evidence of increased angiogenesis or tumor growth among mice compared to wild-type animals.(19)

The *D104N* polymorphism was identified by sequencing of the *COL18A1* gene among patients with Knobloch syndrome, a condition characterized by vitreoretinal degeneration caused by mutation in the gene. The same investigators examined its association with prostate cancer in a hospital-based, case-control study, reporting that carriage of the polymorphic N allele was associated with a 2.5-fold increased risk of prostate cancer compared to those who were homozygous wild-type. They found a similar effect for high grade tumors and did not differ as a function of age at diagnosis.(9) More recent studies, however, have shown no association between the polymorphism in relation to prostate cancer risk overall or for aggressive prostate cancer.(15,20,21)

Although the main effects of the *D104N* genotype were null, we found that men with prostate cancer who carried the variant allele were less likely to be overweight or obese. Moreover, we observed a significant effect modification by body mass index, such that the effect of obesity/overweight on prostate cancer risk and progression depended on the *D104N* genotype. Adipose tissue produces pro- and anti-angiogenic factors, including endostatin. Indeed, individuals who are overweight or obese express higher levels of endostatin compared to healthy weight men,(22) while in an animal model, adipose tissue mass responds and regresses to exogenous angiogenesis inhibitors such as endostatin(12). Thus, the *D104N* polymorphism may impact prostate cancer progression via its modulation of adipose tissue.

One weaknesses of our study is the lack of comprehensive assessment of the endostatin gene, and thus, we cannot exclude that genetic variation across endostatin may be related to prostate cancer risk or progression. Moreover, our study did not focus on polymorphisms in other genes that modulate endostatin secretion or angiogenesis that could still impact overall prostate cancer incidence or progression. Still, the *D104N* is a missense mutation which impacts protein confirmation, and thus our study was hypothesis driven. Our study was focused on Caucasian men, and thus it is unclear whether our findings can be generalized to other populations such as African-American men who have a substantially higher risk of prostate cancer. In addition, we cannot confirm or refute a potential role of population stratification on our study findings.

The present study has a number of important strengths. The large size of our study, including a significant number of advanced stage or high grade cancers, permitted us to examine associations within important subgroups of clinical and tumor characteristics. Moreover, we were able to relate the *D104N* genotype to both prostate cancer incidence and mortality to look at the full spectrum from disease initiation to progression. Our study was the first to evaluate potential effect of the *D104N* genotype according to body mass index. Additional studies are needed to confirm or refute the apparent synergistic associations between the *D104N* genotype and body mass index on prostate cancer development and progression, as well as clarify the potential mechanisms by which this polymorphism could differentially influence prostate cancer outcomes according to degree of adiposity.

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Table 1

Baseline characteristics of prostate cancer cases and controls in the Physicians' Health Study 1982-1995

Characteristic	Prostate Cancer Cases	Controls
N	544	678
Mean age at baseline, years (±SD)	61.2 (±7.5)	61.4 (±7.6)
Mean age at diagnosis, years (±SD)	68.9 (±6.7)	N/A
Mean post-diagnostic follow-up, years (±SD)	12.2 (±5.4)	N/A
Smoking Status in 1982, N (%)		
Never	250 (46.0)	304 (44.8)
Former	252 (46.3)	318 (46.9)
Current	42 (7.7)	56 (8.3)
Body mass index in 1982, N (%)		
<25 kg/m^2	305 (56.1)	423 (62.4)
25-30 kg/m^2	220 (40.4)	237 (35.0)
30+ kg/m^2	19 (3.5)	18 (2.6)
Endostatin Genotype, N (%)		
DD	448 (82.4)	571 (84.2)
DN	90 (16.5)	98 (14.5)
NN	6 (1.1)	9 (1.3)
Tumor Stage, N (%)		
T1/T2	366 (71.3)	N/A
T3/T4N1/M1	147 (28.7)	N/A
Gleason Score, N (%)		
2-6	234 (51.2)	N/A
7	148 (32.4)	N/A
8-10	75 (16.4)	N/A

N/A = Information is not applicable for controls

Table 2

Endostatin *D104N* polymorphism in relation to prostate cancer risk and survival, the Physicians' Health Study, 1982-2007

	NN/DN	DD	NN/DN vs. DD (REF)
	Genotype, N (%)		Relative risk (95% CI)[1]
***D104N* polymorphism and prostate cancer risk**			
Controls	107 (15.8)	571 (84.2)	--
Prostate Cancer Cases	96 (17.6)	448 (82.4)	1.2 (0.9-1.6)
Stage at diagnosis			
T3/N1/M1	20 (13.6)	127 (86.4)	0.8 (0.5-1.4)
T1/T2	69 (18.8)	297 (81.2)	1.3 (0.9-1.8)
Grade at diagnosis			
High grade tumor	34 (15.2)	189 (84.8)	1.0 (0.7-1.5)
Low grade tumor	44 (18.8)	190 (81.2)	1.3 (0.9-2.0)
***D104N* polymorphism and prostate cancer survival**			
	Prostate cancer deaths or mets, N (%)		Hazard ratio (95% CI)[2]
All prostate cancer cases	24 (25.0)	105 (23.4)	1.2 (0.7-1.8)
Stage at diagnosis			
T3/N1/M1	9 (45.0)	59 (46.5)	1.0 (0.5-2.1)
T1/T2	13 (18.8)	45 (15.2)	1.1 (0.6-2.1)

[1] Relative risk from unconditional logistic regression models, adjusted for matching factors age, smoking status, and follow-up time

[2] Hazard ratios (95% Confidence Intervals) from Cox proportional hazard models among cases only (N=544), adjusted for age at diagnosis, tumor grade and stage

Table 3

Effect of body mass index on prostate cancer risk and survival, according to endostatin *D104N* genotype, Physicians' Health Study 1982-2007

	Relative risk (95% CI)		
	NN/DN genotype	**DD genotype**	**P for interaction**
Prostate Cancer Risk [1]			
Overweight/Obese	0.9 (0.5-1.5)	1.6 (1.2-2.1)	<0.001
Healthy weight	1.8 (1.2-2.6)	REF	
Prostate Cancer Survival [2]			
Overweight/Obese	1.2 (0.5-3.0)	1.7 (1.2-2.5)	0.08
Healthy weight	1.7 (1.0-2.9)	REF	

[1] Relative risk from unconditional logistic regression models, adjusted for matching factors age, smoking status, and follow-up time

[2] Hazard ratios (95% Confidence Intervals) from Cox proportional hazard models among cases only, adjusted for age at diagnosis, tumor grade and stage