ORIGINAL MANUSCRIPT

Tumor expression of adiponectin receptor 2 and lethal prostate cancer

Jennifer R. Rider¹, Michelangelo Fiorentino³, Rachel Kelly, Travis Gerke, Kristina Jordahl, Jennifer A. Sinnott, Edward L. Giovannucci^{1,2}, Massimo Loda⁵, Lorelei A. Mucci¹ and Stephen Finn⁶; On behalf of the Transdisciplinary Prostate Cancer Partnership (ToPCaP)

¹Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA, ²Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA, ³Pathology Unit, Addarii Institute of Oncology, S. Orsola-Malpighi Hospital, 40126 Bologna, Italy, ⁴Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA, ⁵Department of Pathology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA and ⁶Department of Pathology, Trinity College Dublin, Dublin 8, Ireland

*To whom correspondence should be addressed. Tel: +1 617 432 3040; Fax: +1-617 566 7805; Email: rider@hsph.harvard.edu

Abstract

To investigate the role of adiponectin receptor 2 (AdipoR2) in aggressive prostate cancer we used immunohistochemistry to characterize AdipoR2 protein expression in tumor tissue for 866 men with prostate cancer from the Physicians' Health Study and the Health Professionals Follow-up Study. AdipoR2 tumor expression was not associated with measures of obesity, pathological tumor stage or prostate-specific antigen (PSA) at diagnosis. However, AdipoR2 expression was positively associated with proliferation as measured by Ki-67 expression quartiles (P -trend < 0.0001), with expression of fatty acid synthase (P -trend = 0.001), and with two measures of angiogenesis (P -trend < 0.1). An inverse association was observed with apoptosis as assessed by the TUNEL assay (P -trend = 0.006). Using Cox proportional hazards regression and controlling for age at diagnosis, Gleason score, year of diagnosis category, cohort and baseline BMI, we identified a statistically significant trend for the association between quartile of AdipoR2 expression and lethal prostate cancer (P -trend = 0.02). The hazard ratio for lethal prostate cancer for the two highest quartiles, as compared to the two lowest quartiles, of AdipoR2 expression was 1.9 (95% confidence interval [CI]: 1.2–3.0). Results were similar when additionally controlling for categories of PSA at diagnosis and Ki-67 expression quartiles. These results strengthen the evidence for the role of AdipoR2 in prostate cancer progression.

Introduction

Adipose tissue functions not only as a fat storage parenchyma, but also as an endocrine and immunological organ (1). One of the most abundant adipocytokines secreted exclusively by adipose tissue is adiponectin, a protein hormone that modulates several metabolic processes, including glucose regulation and fatty acid catabolism (2,3). Somewhat paradoxically, a strong inverse relationship exists between the degree of adiposity and circulating adiponectin levels (4). Functionally, adiponectin serves as an insulin-sensitizing adipokine by stimulating the phosphorylation and activation of 5'-adenosine monophosphate-activated

protein kinase (5), inhibiting inflammation and angiogenesis. Consequently, it is hypothesized to limit tumor invasiveness.

Laboratory and population-based data support the role of adiponectin in tumor development, and in prostate carcinogenesis, specifically. Prostate cancer cell line studies suggest that adiponectin decreases oxidative stress through a receptor-dependent mechanism (6), and in both androgen-dependent and -independent cell lines, adiponectin has been observed to inhibit cell growth (7). Within the Physicians' Health Study (PHS), one of the cohorts utilized for the present analysis, circulating

Abbreviations

AdipoR	adiponectin receptor
BMI	body mass index
CI	confidence interval
FASN	fatty acid synthase
HR	hazard ratios
HPFS	Health Professionals Follow-up Study
PHS	Physicians' Health Study
PSA	prostate-specific antigen
TMA	tissue microarrays

prediagnostic adiponectin levels were found to be inversely associated with development of lethal prostate cancer (8). Multiple studies of germ-line variation in adiponectin-related genes also suggest an association with prostate cancer (9–11). A study nested within the PHS (9), found four polymorphisms in ADIPOQ that were significantly associated with overall prostate cancer risk, two of which were also associated with circulating prediagnostic adiponectin levels (rs266729 and rs182052). In a USA-based case-control study, Kaklamani *et al.* (11) also found that single nucleotide polymorphisms in ADIPOQ, including rs266729, were associated with prostate cancer risk; these SNPs were previously found to be associated with colon and breast cancer risk in the same direction. A meta-analysis conducted by Fan *et al.* (10) of three ADIPOQ SNPs previously investigated with respect to multiple cancer types found that rs1501299 was consistently associated with prostate cancer; a recent study found that rs1501299 was also associated with adiponectin levels in the consistent direction (12).

While there is substantial evidence that adiponectin is implicated in cancer development, less is understood about the cancer-related role of the primary physiologically active, transmembrane receptors for adiponectin, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). The receptors are believed to be functionally distinct with respect to downstream pathway activation. To our knowledge, only one small study of protein expression of AdipoRs in prostate tumor tissue has been published to date. In that study of 72 prostate cancer cases and 27 non-cases (11 patients with benign prostatic hyperplasia and 16 healthy controls) (13), lower protein expression of AdipoR1 and AdipoR2 was observed in prostate tissue from cases compared to non-cases, but no statistically significant associations were observed with clinicopathological characteristics. For other tumor sites, both positive and inverse associations between the AdipoR expression and carcinogenesis have been reported. Higher expression of both receptors has been associated with more favorable disease characteristics in thyroid carcinoma (14), and with a decreased risk of tumor invasiveness in colorectal carcinoma (15,16). However, other studies of colorectal carcinoma have demonstrated a positive association between AdipoR2 expression with tumor, node and metastasis stage (17). Higher AdipoR1 and AdipoR2 expression have been shown to be associated with invasion, metastases and poor survival in adenocarcinoma, gastric cancer and breast cancer (18–20).

We hypothesized that the local effects of adiposity and adiponectin on prostate cancer progression were mediated in part by variability in prostate tumor expression of AdipoR2, a receptor for the biologically active high molecular weight form of adiponectin. We comprehensively investigated the association between tumor expression of AdipoR2, with respect to metabolic syndrome risk factors, clinical characteristics and time to development of distant metastases or prostate cancer-specific death (subsequently termed *lethal prostate cancer*) using data compiled prospectively for 866 men with prostate cancer from the PHS and the Health Professionals Follow-Up Study (HPFS).

Materials and methods

Study population

The present study is nested among men diagnosed with prostate cancer who are participants in the PHS and the HPFS cohorts. The PHS (21–23) was initiated as a randomized, double-blind, placebo-controlled trial for the primary prevention of cardiovascular disease and cancer. The study was conducted in two phases; in PHS I, initiated in 1982, men were randomly assigned to aspirin and β -carotene, and in PHS II, initiated in 1997, men were randomly assigned to vitamin E, C and a multivitamin. In total, in USA, 29067 healthy male physicians aged 40–84 years at baseline were recruited. Circulating levels of adiponectin are available for 14916 (51%) of these men measured in blood samples taken before randomization. The majority of participants were Caucasian (94%). Participants are followed through regular questionnaires to collect data on diet, health and lifestyle behaviors and medical history. Compliance and health endpoints including prostate cancer are assessed biannually.

The HPFS was initiated in 1986, when 51529 male health professionals aged 40–75 years completed a mailed questionnaire on demographic characteristics, risk factors, preventive behaviors, diet and use of supplements. The cohort is predominantly Caucasian (>91%). Exposure information and medical events, including prostate cancer, are updated through biennial follow-up using mailed questionnaires.

The present study includes men with histologically confirmed prostate cancer diagnosed between 1983 and 2004. Clinical and pathological data related to the cancer diagnosis, including age at diagnosis, prostate-specific antigen (PSA) levels and tumor stage are collated through systematic medical record review. Post-diagnosis, prostate cancer cases are followed (annually in PHS, biennially in HPFS) through questionnaires to collect information on their prostate cancer clinical course, including development of metastases. Cancer-specific and all-cause mortality is ascertained through repeated mailings and telephone calls to participants, as well as periodic searches of the National Death Index. Cause of death is assigned based on review of death certificates, information from the family and medical records. Follow-up for metastases and mortality is available through 1 March 2011 in the PHS (>99% complete) and 31 December 2011 in the HPFS (>98% complete).

Tumor collection and immunohistochemistry

We retrieved original tumor specimens (blocks, as well as hematoxylin and eosin slides) from the diagnosing institution for men in PHS or HPFS who underwent radical prostatectomy (95%) or transurethral resection of the prostate (5%). Our pathology team undertook a standardized review of the original hematoxylin and eosin slides from the referring hospitals for primary and secondary Gleason grade (24), blinded to the original pathology reports and any clinical data. For immunohistochemical evaluation, we constructed high-density tissue microarrays (TMAs). Areas of tumor tissue were identified on the hematoxylin and eosin slides, and at least three 0.6mm cores were removed from each block and embedded in the TMA paraffin block using a manual arrayer. For a subset of the cases, adjacent morphologically normal prostate tissue was also included on the arrays. The 866 cases (555 from HPFS and 311 from PHS) were included on 9 TMAs.

Sections of 4 μ m in thickness were cut from each TMA on charged slides and used for immunohistochemical analysis. Sections were incubated using a rabbit polyclonal antibody for AdipoR2 (Phoenix Pharmaceuticals, Burlingame, CA, diluted 1:200 in phosphate-buffered saline). Antigen retrieval for AdipoR2 was performed in microwave for 5min in citrate buffer. Antigen-antibody reactions were revealed with standardized development times using the avidin-biotin complex method with diaminobenzidine as substrate on an automated Biogenex i6000 immunostainer (Biogenex, San Ramon, CA). LNCap prostate cancer cell lines were used as a positive control for immunohistochemistry, whereas normal isotype serum was used as a negative control.

AdipoR2 protein expression was scored semi-quantitatively on images scanned into the Java-based TMAJ software (25). The pathologists (S.F. and M.L.) were blinded to clinical and pathological data. Representative staining is illustrated in Figure 1. The cytoplasmic expression level of AdipoR2 was measured by two variables: (i) *quantity*, defined as the percentage of positive cells—0 (negative), 1 (1–25% cells positive), 2 (26–50%), 3 (51–75%) and 4 (76–100%); and (ii) *staining intensity*, defined qualitatively—0

Figure 1. Representative images of TMA cores stained with AdipoR2 antibody. Panels A (Gleason 4 + 4) and B (Gleason 3 + 4) both show 3+ intensity staining in 100% of tumor cells. Panel B highlights strong stromal staining commonly seen in AdipoR2 IHC (arrows). Panel C (Gleason 4 + 3) shows 2+ intensity and Panel D (Gleason 3 + 3) shows 1+ intensity.

(negative), 1 (weak), 2 (moderate) and 3 (intense). These were combined using the product of quantity and staining intensity, a variation of the Allred scoring method (26).

The degree of cell proliferation was evaluated on TMAs using Ki67 antigen, which is expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase) and is absent in resting (G0) cells. Five micrometer sections of TMAs were used for the Ki67 antigen. We used a rabbit polyclonal antibody (Vector, Burlingame, CA) diluted 1:1500 with citrate-based antigen retrieval. The Ki67 score was assessed as the number of stained nuclei over the total number of tumor nuclei using the Ariol instrument SL-50 (Applied Imaging, Grand Rapids, MI) after selection of the tumor epithelial areas of each core for full quantitative image analysis.

Fatty acid synthase (FASN) protein expression (27) and measures of apoptosis using the TUNEL assay (28) were evaluated as described previously. The following measures of angiogenesis were additionally investigated among men in the HPFS cohort only ($n = 555$): (i) *microvessel density*, the number of vascular structures in a high-power field ($\times 200$); (ii) *vessel area* in μm^2 and (iii) *vessel irregularity*, calculated as the vessel lumen perimeter²/ $4\pi \times \text{area}$, where a value of 1.0 is a perfect circle and values >1.0 indicate increasing irregularity, using previously described methodology (29).

Circulating adiponectin levels

Plasma adiponectin levels were available for a subset of men in the PHS ($N = 90$) who provided a baseline blood sample and were included in a previously published nested case-control study on circulating adiponectin levels and prostate cancer risk (8). Full-length adiponectin concentrations were measured by competitive radioimmunoassay (Linco Research, St Charles, MO) in the laboratory of Dr Nader Rifai (Children's Hospital, Boston, MA), which demonstrated a coefficient of variation of 3.4%. We have previously demonstrated a single blood measurement of adiponectin is reasonably accurate and stable over time; two adiponectin measurements over a 1-year period had a body mass index (BMI)-adjusted intraclass correlation of 0.84 (95% confidence interval [CI]: 0.65–0.94) (30).

Statistical methods

For each participant, the product of AdipoR2 quantity and staining intensity was averaged across all of their tumor cores and then divided into quartiles. This same approach was conducted separately across adjacent normal cores. We investigated relationships between AdipoR2 protein expression in tumor tissue with BMI at baseline, waist circumference and prediagnostic circulating adiponectin levels. In addition, we compared AdipoR2 tumor expression to other features including Gleason score, Ki67, FASN, PSA at diagnosis, pathologic tumor stage and measures of angiogenesis and apoptosis. Because Ki67 and FASN expression were evaluated by the Ariol system and apoptosis was assessed using the TUNEL assay, the quartiles of Ki-67, FASN and apoptosis were ranked by TMA to control for potential batch effects. For AdipoR2 and all covariates, categories were treated as continuous to evaluate a test for trend using ANOVA. All relationships were evaluated in the HPFS, the PHS and the combined cohort with three exceptions: angiogenesis was measured only in the HPFS cohort, prediagnostic circulating adiponectin was measured only in a subset of men with available blood from the PHS ($N = 90$) and waist circumference was evaluated only in the combined cohort due to the small total sample size with available data ($n = 669$).

The prespecified primary analysis involved examining quartiles of AdipoR2 expression with respect to lethal prostate cancer. For univariate analyses, the Kaplan–Meier method was used to estimate the cumulative incidence of lethal prostate cancer (defined as development of distant bone or organ metastases, or cancer-specific mortality) in the low and high AdipoR2 expression groups; log-rank tests were used to test strata equality. We further examined the association between tumor AdipoR2 expression and lethal prostate cancer by using Cox proportional hazards regression to calculate adjusted hazard ratios (HR) and 95% CIs. Follow-up time was censored if the participant experienced death from a cause other than prostate cancer or survived until the end of follow-up date (PHS: 1 March 2011; HPFS: December 2011). Multivariate survival models included variables selected *a priori*: cohort, age at diagnosis (continuous), calendar year of diagnosis (1983–1989, 1990–1992, 1993–2004), BMI at baseline (continuous) and Gleason score (continuous); additional models also

adjusted for PSA at diagnosis (<4 ng/ml, 4–<10 ng/ml and ≥10 ng/ml) and Ki67 expression (quartiles). A P value for trend for AdipoR2 expression was estimated for each model by including quartile of expression as a continuous variable. A likelihood-ratio test was used to determine if the high AdipoR2 expression indicator significantly improved model fit in the multivariate models.

All analyses were undertaken using the SAS Statistical Analysis Software (Version 9.3). The research protocol was approved by the institutional review board at Partners Healthcare and the Harvard T.H. Chan School of Public Health.

Results

Characteristics of the individual and combined patient cohorts are shown in Table 1. The median age at prostate cancer diagnosis was 66 years. Although obesity (BMI ≥ 30 kg/m²) was uncommon, more than 41% of the men were classified as overweight (BMI: 25–30 kg/m²). Most men were diagnosed with localized tumors, and approximately 62% of tumors were graded as Gleason score 7. Over a median of 13.6 years of follow-up, 94 lethal prostate cancer events occurred.

Among men with cores of both cancer and adjacent morphologically normal tissue (*n* = 141), the AdipoR2 expression levels in normal and tumor areas were positively correlated (Pearson ρ = 0.39; *P* < 0.0001). We did not find a significant association between AdipoR2 expression in tumor or normal tissue and BMI at baseline in the combined cohort (tumor tissue *P*-value = 0.33; normal tissue *P*-value = 0.52), or in either of the individual cohorts. Similarly, there was no association between AdipoR2 expression in tumor or normal tissue and waist circumference at the time of diagnosis in the combined cohort (tumor tissue *P*-value = 0.21; normal tissue *P*-value = 0.66). In a subset of 90 men from the PHS with prediagnostic circulating adiponectin

data, the correlation between circulating adiponectin levels and AdipoR2 tumor expression was weak and was neither statistically significant in the univariate analyses (ρ = 0.02; *P* = 0.84) nor in the multivariable analyses adjusting for age at diagnosis, Gleason score, year of diagnosis and BMI at baseline (*P* = 0.75).

Table 2 describes the relationship between AdipoR2 expression in tumor tissue and selected clinical indices. There was some suggestion of increasing AdipoR2 expression across categories of Gleason ≤6, 3 + 4, 4 + 3 and 8–10 tumors in the combined (*P*-trend = 0.03) and HPFS (*P*-trend = 0.07) cohorts, but the association was not apparent in the PHS (*P*-trend = 0.80). There was no association with tumor stage in the combined, HPFS or PHS cohort (*P*-trend = 0.28, 0.59, 0.69, respectively).

There was a suggestion of increasing AdipoR2 tumor expression with increasing number of vessels quartile (*P*-trend = 0.05) and vessel irregularity quartile (*P*-trend = 0.07), but no association with vessel area quartiles (*P*-trend = 0.99). It should be noted that these three measures of angiogenesis were significantly correlated in this population (*P* < 0.03 for each pairwise comparison). Expression levels were strongly and positively associated with cellular proliferation across categories of Ki-67 expression quartiles in the combined cohort (*P*-trend < 0.0001), in the HPFS cohort (*P*-trend < 0.0001) and in the PHS cohort (*P*-trend = 0.04). Conversely, expression levels were inversely associated with apoptosis as measured by the TUNEL assay (*P*-trend = 0.006 in the combined cohort, 0.064 in HPFS and 0.067 in PHS). Similarly, there was a significant, positive association between continuous AdipoR2 expression and FASN protein expression quartiles in the combined, HPFS and PHS cohorts (*P*-trend = 0.001, 0.01 and 0.01, respectively). There was no association with expression across categories of BMI at baseline, BMI at diagnosis or waist circumference at diagnosis. Additionally, AdipoR2 expression in

Table 1. Selected characteristics of prostate cancer cases with tumor tissue in the HPFS and PHS tumor cohorts

	HPFS (N = 555)	PHS (N = 311)	Combined cohort (N = 866)
Median age at diagnosis, years (IQR)	66.0 (62.0–70.0)	66.3 (62.2–70.1)	66.0 (62.0–70.0)
Median follow-up time, years (IQR)	14.3 (11.8–17.3)	11.3 (8.9–15.0)	13.6 (10.1–16.4)
BMI at baseline, kg/m ² (%)			
<25	267 (48.1)	177 (56.9)	444 (51.3)
25–30	238 (42.9)	122 (39.2)	360 (41.6)
≥30	36 (6.5)	12 (3.9)	48 (5.5)
Missing	14 (2.5)	0 (0)	14 (1.6)
Waist circumference (%)			
≤36	160 (28.8)	75 (24.1)	235 (27.1)
36–39	132 (23.8)	64 (20.6)	196 (22.6)
>39	160 (28.8)	78 (25.1)	238 (27.5)
Missing	103 (18.6)	94 (30.2)	197 (22.8)
Pathological TNM stage (%)			
T1/T2	383 (69.0)	233 (74.9)	616 (71.1)
T3	148 (26.7)	60 (19.3)	208 (24.0)
T4 or N1 or M1	24 (4.3)	14 (4.5)	38 (4.4)
Missing	0 (0)	4 (1.3)	4 (0.5)
Gleason score (%)			
2–6	87 (15.7)	78 (25.1)	165 (19.1)
3+4	209 (37.7)	114 (36.7)	323 (37.3)
4+3	151 (27.2)	60 (19.3)	211 (24.4)
8–10	108 (19.5)	59 (19.0)	167 (19.3)
PSA at diagnosis, ng/ml (%)			
<4	56 (10.1)	33 (10.6)	89 (10.3)
4 to <10	262 (47.2)	175 (56.3)	437 (50.5)
≥10	150 (27.0)	59 (19.0)	209 (24.1)
Missing	87 (15.7)	44 (14.2)	131 (15.1)
Lethal prostate cancer outcomes (%)	63 (11.4)	31 (10.0)	94 (10.9)

IQR, interquartile range.

Table 2. Association between AdipoR2 expression and selected clinical characteristics

Covariates	AdipoR2 expression			P-trend
	n	M	SD	
BMI at baseline ^a				
<25	444	6.685	2.848	0.458
25–30	360	6.715	2.899	
≥30	48	6.111	2.760	
BMI at diagnosis				
<25	260	7.322	2.800	0.482
25–30	249	7.069	2.877	
≥30	45	7.211	2.755	
Waist circumference at diagnosis				
≤30	235	7.007	2.816	0.128
36–39	196	6.805	3.048	
>39	238	6.605	2.785	
Gleason grade				
2–6	165	6.190	2.877	0.031
3+4	323	6.814	2.924	
4+3	211	6.493	2.770	
8–10	167	7.095	2.824	
Tumor stage				
T2	616	6.604	2.925	0.283
T3	208	6.848	2.726	
T4/N1/M1	38	6.862	2.840	
Ki-67 expression				
Q1	241	6.053	2.674	<0.0001
Q2	162	6.649	2.802	
Q3	210	6.755	2.881	
Q4	197	7.371	2.964	
FASN protein expression				
Q1	199	6.439	2.974	0.001
Q2	210	6.147	2.717	
Q3	218	7.078	2.799	
Q4	203	7.089	2.896	
TUNEL assay: % stained area ^a				
Q1	183	6.880	2.882	0.006
Q2	193	6.900	2.846	
Q3	132	6.322	2.662	
Q4	159	6.177	2.858	
Number of vessels				
Q1	96	6.997	2.805	0.052
Q2	104	6.668	2.522	
Q3	111	7.155	2.848	
Q4	103	7.628	2.968	
Vessel irregularity				
Q1	98	7.303	3.005	0.074
Q2	109	7.451	2.746	
Q3	107	6.983	2.716	
Q4	101	6.726	2.725	
Vessel area				
Q1	104	7.061	2.751	0.990
Q2	111	7.297	2.842	
Q3	102	6.900	2.804	
Q4	98	7.206	2.828	

^aMeasures of angiogenesis and BMI at baseline; available for HPFS participants only.

tumor tissue was not associated with PSA levels at diagnosis in univariable linear regression models ($\beta = 0.002$; P -value = 0.60).

As shown in Table 3, quartiles of AdipoR2 expression were evaluated in an unadjusted and in three multivariable Cox proportional hazards models: (i) controlling for age at diagnosis, Gleason score, year of diagnosis category and cohort; (ii)

additionally controlling for BMI at baseline and 3) additionally controlling for Ki-67 expression quartile. The HRs for AdipoR2 expression were increased in the upper two quartiles relative to the lower two quartiles and were similar across the crude and multivariable models, but did not achieve statistical significance in any of the models. However, there was a trend of increasing rates of lethal prostate cancer with increasing expression of AdipoR2 in the unadjusted (P -trend = 0.07) and the multivariable models, which reached statistical significance in Model 2 (P -trend = 0.02) but not in Model 1 (P -trend = 0.08) or Model 3 (P -trend = 0.10). In analyses that separately examined the individual components of the AdipoR2 expression score, both staining intensity and staining quantity appear to contribute to positive associations with lethal prostate cancer (Table 3).

Given similar HR estimates for lethal prostate cancer for the upper quartiles of AdipoR2 expression, we dichotomized AdipoR2 expression at the median. The results for all multivariable Models 1–3 were statistically significant and similar to the crude results (HR: 1.7; 95% CI: 1.1–2.6). A likelihood ratio test comparing Model 3, which included all the predictor variables, to the nested model without the high versus low AdipoR2 expression indicator showed that accounting for AdipoR2 expression significantly improved model fit (P -value = 0.01). The cumulative incidence curves for high and low AdipoR2 expression in the combined cohort are shown in Figure 2. The log-rank test of equality over the high and low AdipoR2 expression strata indicated a statistically significant difference between the curves with a P -value of 0.01.

When we evaluated Model 3 for lethal prostate cancer within strata of BMI (BMI < 25 versus BMI ≥ 25), the HR for AdipoR2 expression appeared stronger in the overweight men (HR: 2.28; 95% CI: 1.18–4.37) than in the healthy weight men (HR: 1.17; 95% CI: 0.60–2.28). However, the P -interaction for modification of the effect of AdipoR2 by BMI was not statistically significant (P -interaction = 0.16). Among men with information on PSA at diagnosis ($n = 679$), there were 52 lethal events and the HR was somewhat attenuated (HR: 1.5; 95% CI: 0.8–2.8), but did not change when PSA category was added to the model (HR: 1.5; 95% CI: 0.8–2.7). Sensitivity analyses that restricted to Caucasian men or men treated with radical prostatectomy identified results similar to the unrestricted analyses (results not shown).

Discussion

In this study of men with prostate cancer, we identified a significant positive association between AdipoR2 expression and measures of tumor aggressiveness. Furthermore, we observed a nearly 2-fold increase in risk of prostate cancer death when comparing high to low tumor expression of AdipoR2. We identified no consistent associations between AdipoR2 expression levels and tumor stage or grade at the time of surgery, suggesting that high AdipoR2 expression could be a marker of micrometastases.

The evidence in the literature for the relationship between the expression of AdipoR2 and carcinogenesis is inconsistent. In the only study of prostate cancer, AdipoR2 expression was reported to be lower in prostate tumors (13), but no statistically significant associations were observed with clinicopathological characteristics. Given that circulating adiponectin has been shown to be inversely associated with high-grade risk and lethality of prostate cancer in PHS (8), it may be hypothesized that an increase in expression of its receptors in malignant cells would also be associated with a decrease in risk. The suppressive effects of adiponectin, thought to be mediated through a reduction in reactive oxidative species and a mediation of

Table 3. HR (95% CI) for lethal prostate cancer according to AdipoR2 expression

AdipoR2 measure	n total	n events	Unadjusted		Adjusted model 1		Adjusted model 2		Adjusted model 3	
			HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
Expression score	140	12	1.00	(Ref)	1.00	(Ref)	1.00	(Ref)	1.00	(Ref)
	290	23	0.86	0.43–1.73	0.76	0.37–1.53	0.67	0.32–1.39	0.76	0.36–1.60
	237	34	1.64	0.85–3.16	1.35	0.69–2.63	1.44	0.74–2.81	1.64	0.81–3.32
	199	25	1.39	0.70–2.76	1.33	0.65–2.71	1.51	0.74–3.10	1.33	0.62–2.88
Staining quantity	207	16	1.00	(Ref)	1.00	(Ref)	1.00	(Ref)	1.00	(Ref)
	195	21	1.33	0.69–2.55	0.89	0.46–1.74	1.11	0.55–2.24	1.33	0.65–2.73
	192	24	1.49	0.79–2.80	1.44	0.76–2.73	1.68	0.86–3.30	1.68	0.83–3.40
	272	33	1.36	0.75–2.47	1.37	0.72–2.6	1.74	0.88–3.45	1.53	0.76–3.09
Staining intensity	273	19	1.00	(Ref)	1.00	(Ref)	1.00	(Ref)	1.00	(Ref)
	133	12	1.31	0.64–2.71	0.98	0.47–2.04	0.93	0.42–2.06	0.88	0.38–2.01
	258	38	2.23	1.29–3.87	1.82	1.04–3.19	2.07	1.16–3.71	2.20	1.18–4.12
	202	25	1.79	0.99–3.26	1.71	0.93–3.17	2.09	1.1–3.96	1.70	0.84–3.47

Model 1: Controlling for age at diagnosis, Gleason score, year of diagnosis category, cohort.
Model 2: Controlling for variables in Model 1 and also BMI at baseline.
Model 3: Controlling for variables in Model 2 and also Ki67 expression.

Figure 2. Cumulative incidence of lethal prostate cancer with survival tables comparing high and low AdipoR2 expression.

cellular proliferation and apoptosis, may be facilitated by abundant expression of AdipoR2. This would thereby limit cancer development and metastasis, consistent with several studies of other cancer sites (15,16,31).

By contrast but consistent with our data, adiponectin has been shown to have potent pro-angiogenic actions in some studies (32–34), which may explain the strong association we observed between AdipoR2 expression with vascular size and irregularity. These two indicators of angiogenesis have previously been found to be associated with lethal prostate cancer in this cohort (29). Furthermore, adiponectin and its receptors have been demonstrated to direct the migration of prostate cancer cells through the upregulation of integrin expression, a process which has been associated with metastasis (35). Finally, cell line studies have reported that adiponectin and the expression of

its receptors may have supportive effects on cancer cell survival under stress conditions, which is in agreement with our finding of an inverse association between expression and apoptosis. Therefore, it is feasible that high adiponectin receptor expression may result in increased tumor invasion, more aggressive behavior and potentially metastasis (18).

An alternative explanation for our findings is that in a low adiponectin milieu, as has been shown to be associated with an increased risk of prostate cancer and high-grade disease in PHS, adiponectin receptor expression may be upregulated in a compensatory manner. A reciprocal relationship between hormones and their receptors has previously been demonstrated for many other hormonal axes (36–38). It should be noted that an inverse association between adiponectin and risk has not been observed in all studies (39,40). However, given that hypoadiponectinemia,

and therefore the compensatory response, is likely to be greatest in obese patients who are also at greatest risk from prostate cancer death, this may explain the increased HR reported here for those with a high BMI.

Both these theories rely on an association between AdipoR2 expression and adiponectin. Our study did not provide evidence of a strong relationship between prediagnostic circulating adiponectin and tumor expression of AdipoR2 at the time of surgery, which could be attributed to the timing of measurement of circulating adiponectin levels, or alternatively, that AdipoR2 expression within the prostate could be altered independently of circulating adiponectin. Recent studies suggest a third receptor, T-cadherin, encoded by *CDH13*, which may affect circulating adiponectin (41). Further, variants in *ADIPOR2* have been shown to be associated with diabetes prevalence independently of adiponectin concentrations (42). The relationship between AdipoR2 expression and prostate cancer may be similarly independent of adiponectin and instead could be mediated through interactions with other hormones and cytokines, or expression levels may be altered in response to cancer development (20). This latter scenario of an independent effect would be consistent with the lack of association observed between AdipoR2 expression and BMI at baseline or waist circumference at diagnosis, despite these indicators of adiposity being strongly correlated with circulating adiponectin levels. In fact, this finding of no association is in agreement with existing studies which have concluded that other factors, including cancer cell characteristics, may have a greater effect on expression than adiposity (16). With that said, we cannot rule out that BMI and waist circumference insufficiently capture visceral adiposity, which could be related to AdipoR2.

In this study we report for the first time, a strong association between the expression of AdipoR2 and FASN, which has also been associated with lethal prostate cancer in overweight men (27). Both FASN and AdipoR2 play key roles in fatty acid and lipid metabolism, and both have close relationships with insulin; insulin increases expression of FASN in adipocytes, whereas AdipoR2 expression has been shown to be associated with insulin sensitivity (43). Further, overexpression of the adiponectin receptors in animal studies have demonstrated their ability to stimulate fatty acid oxidation via activation of peroxisome proliferator-activated receptor and adenosine monophosphate-activated protein kinase (44,45), whereas FASN is thought to be inversely related to adenosine monophosphate-activated protein kinase activation. These inconsistencies again point to a complex causal pathway and add further evidence for the important role of insulin and lipid metabolism in prostate cancer. Additional work is required to disentangle these pathways and to determine whether the observed relationship between AdipoR2 and FASN plays a crucial role, or whether their expression reflects two independent markers of tumor aggressiveness.

This represents only the second, and the largest, study to date to explore the relationship between prostate cancer and expression of AdipoR2. There are a number of strengths to this study. The nesting within HPFS and PHS confers the benefit of a large sample size with a long follow-up, together with validated data on anthropometric measures, extensive biological and clinical data and well-defined lethal prostate cancer outcomes. Potential bias was minimized through the use of standardized procedures for the collection, storage, processing and assaying of blood samples and for the pathological review which was conducted blind to patient outcome. Multivariate statistical analyses were employed to account for potential confounders including age, stage, grade and PSA levels.

Our study also has important limitations. Only a single, pre-diagnostic adiponectin measurement from the baseline sample was used (mean time between blood draw and diagnosis 8.1 [SD: 3.3] years in HPFS and 13.5 [SD: 5.0] years in PHS), which may not have fully characterized long-term status; this is of particular interest given the lack of association between adiponectin concentrations and the expression of its receptor. Serum adiponectin was only available for a subset of men ($N = 90$), and in this group there was no association between circulating adiponectin and BMI at baseline ($r = -0.12$; $P = 0.16$); BMI at diagnosis ($r = -0.10$; $P = 0.30$) or waist circumference ($r = -0.11$; $P = 0.38$). Further, total serum adiponectin levels were measured, rather than the high molecular weight form, which is thought to be the form that inhibits prostate cancer growth (7). Our study population consisted primarily of Caucasian men, limiting our ability to generalize our findings to other racial and ethnic groups, including African American men who have been found to have lower circulating levels of adiponectin (46–48). Finally, there is a possibility of chance findings due to multiple comparisons. However, the consistency of our results with a number of markers of tumor aggressiveness, as well as with lethal prostate cancer, suggest a true effect that should be further investigated.

The current study focused solely on AdipoR2, despite the potential for other physiologically active receptors, including AdipoR1, to function in the prostate. Having attempted several antibodies and protocols for AdipoR1, not one performed sufficiently for a high-quality histological study when utilizing appropriate positive, negative and isotype controls. Only AdipoR2 passed this quality assessment and only for AdipoR2 was it possible to generate scientifically sound data. However, our inability to incorporate AdipoR1 into the present study represents an important limitation.

To date, the relationship between adiponectin receptor expression and carcinogenesis is somewhat controversial; the potential underlying mechanisms remain unknown and are likely to be tissue-, cell-type and context specific (7,14). Here we add to this body of literature and report that the expression of adiponectin receptors is associated with a worse prognosis in prostate cancer. Our results may have implications for the use of AdipoR agonists in the treatment of diabetes and other obesity-related disorders, which is currently the focus of much research (49,50). Our study also complements the existing literature by indicating an additional link between adiposity and prostate cancer mortality. Further elucidation of the role of adiponectin receptors and their interplay with BMI, adiponectin, FASN and other cytokines (13) will help to shed light on prostate cancer etiology and the unknown nature of its progression. The potential utility of AdipoR2 tumor expression as a marker of clinically relevant disease, and its ability to better identify and classify high-risk patients, should also be explored.

Funding

Dana-Farber/Harvard Cancer Center Specialized Programs of Research Excellence program in Prostate Cancer (5P50CA090381-08); the National Cancer Institute (T32 CA009001, CA55075, CA141298, CA13389, CA-34944, CA-40360, CA-097193 and PO1 CA055075); the National Heart, Lung, and Blood Institute (HL-26490 and HL-34595). J.R.R., L.A.M. and S.F. are Prostate Cancer Foundation Young Investigators.

Acknowledgements

We are grateful to the participants and staff of the Physicians' Health Study and Health Professionals Follow-Up Study for

their valuable contributions. We are also grateful to Carson Smith for her help with manuscript preparation. In addition, we would like to thank the following state cancer registries for their cooperation and assistance: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The Dana-Farber/Harvard Cancer Center Tissue Microarray Core Facility constructed the tissue microarrays in this project, and we would like to thank Chungdak Li for expert tissue microarray construction.

Conflict of Interest statement: None declared.

References

- de Ferranti, S. et al. (2008) The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin. Chem.*, 54, 945–955.
- Scherer, P.E. et al. (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.*, 270, 26746–26749.
- Berg, A.H. et al. (2002) ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol. Metab.*, 13, 84–89.
- Cnop, M. et al. (2003) Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia*, 46, 459–469.
- Yamauchi, T. et al. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.*, 8, 1288–1295.
- Lu, J.P. et al. (2012) Adiponectin inhibits oxidative stress in human prostate carcinoma cells. *Prostate Cancer Prostatic Dis.*, 15, 28–35.
- Bub, J.D. et al. (2006) Adiponectin as a growth inhibitor in prostate cancer cells. *Biochem. Biophys. Res. Commun.*, 340, 1158–1166.
- Li, H. et al. (2010) A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. *Clin. Chem.*, 56, 34–43.
- Dhillon, P.K. et al. (2011) Common polymorphisms in the adiponectin and its receptor genes, adiponectin levels and the risk of prostate cancer. *Cancer Epidemiol. Biomarkers Prev.*, 20, 2618–2627.
- Fan, H.J. et al. (2013) Three adiponectin rs1501299G/T, rs822395A/C, and rs822396A/G polymorphisms and risk of cancer development: a meta-analysis. *Tumour Biol.*, 34, 769–778.
- Kaklamani, V. et al. (2011) Polymorphisms of ADIPOQ and ADIPOR1 and prostate cancer risk. *Metabolism*, 60, 1234–1243.
- de Faria, A.P. et al. (2014) Adiponectin 11377C/G and +276G/T polymorphisms affect adiponectin levels but do not modify responsiveness to therapy in resistant hypertension. *Basic Clin. Pharmacol. Toxicol.* doi:10.1111/bcpt.12368. [Epub ahead of print].
- Michalakis, K. et al. (2007) Serum adiponectin concentrations and tissue expression of adiponectin receptors are reduced in patients with prostate cancer: a case control study. *Cancer Epidemiol. Biomarkers Prev.*, 16, 308–313.
- Cheng, S.P. et al. (2013) Expression and biologic significance of adiponectin receptors in papillary thyroid carcinoma. *Cell Biochem. Biophys.*, 65, 203–210.
- Hiyoshi, M. et al. (2012) Adiponectin receptor 2 is negatively associated with lymph node metastasis of colorectal cancer. *Oncol. Lett.*, 3, 756–760.
- Byeon, J.S. et al. (2010) Adiponectin and adiponectin receptor in relation to colorectal cancer progression. *Int. J. Cancer*, 127, 2758–2767.
- Gialamas, S.P. et al. (2011) Serum adiponectin levels and tissue expression of adiponectin receptors are associated with risk, stage, and grade of colorectal cancer. *Metabolism*, 60, 1530–1538.
- Shin, E. et al. (2013) Adiponectin receptor expression in gastric carcinoma: implications in tumor development and progression. *J. Cancer Res. Clin. Oncol.*, 139, 709–718.
- Jeong, Y.J. et al. (2011) Expression of leptin, leptin receptor, adiponectin, and adiponectin receptor in ductal carcinoma in situ and invasive breast cancer. *J. Breast Cancer*, 14, 96–103.
- Howard, J.M. et al. (2010) Associations between leptin and adiponectin receptor upregulation, visceral obesity and tumour stage in oesophageal and junctional adenocarcinoma. *Br. J. Surg.*, 97, 1020–1027.
- Christen, W.G. et al. (2000) Design of Physicians' Health Study II—a randomized trial of beta-carotene, vitamins E and C, and multivitamins, in prevention of cancer, cardiovascular disease, and eye disease, and review of results of completed trials. *Ann. Epidemiol.*, 10, 125–134.
- Steering Committee of the Physicians' Health Study Research Group. (1989) Final Report on the Aspirin Component of the Ongoing Physicians' Health Study. *N. Engl. J. Med.*, 321, 129–135.
- Hennekens, C.H. et al. (1996) Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N. Engl. J. Med.*, 334, 1145–1149.
- Stark, J.R. et al. (2009) Gleason score and lethal prostate cancer: does 3 + 4 = 4 + 3? *J. Clin. Oncol.*, 27, 3459–3464.
- Faith, D.A. et al. (2004) Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays. *Prostate*, 61, 215–227.
- Allred, D.C. et al. (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod. Pathol.*, 11, 155–168.
- Nguyen, P.L. et al. (2010) Fatty acid synthase polymorphisms, tumor expression, body mass index, prostate cancer risk, and survival. *J. Clin. Oncol.*, 28, 3958–3964.
- Zu, K. et al. (2014) Dietary lycopene, angiogenesis, and prostate cancer: a prospective study in the prostate-specific antigen era. *J. Natl Cancer Inst.*, 106, djt430.
- Mucci, L.A. et al. (2009) Prospective study of prostate tumor angiogenesis and cancer-specific mortality in the health professionals follow-up study. *J. Clin. Oncol.*, 27, 5627–5633.
- Pischon, T. et al. (2003) Adiponectin: stability in plasma over 36 hours and within-person variation over 1 year. *Clin. Chem.*, 49, 650–652.
- Kelesidis, I. et al. (2006) Adiponectin and cancer: a systematic review. *Br. J. Cancer*, 94, 1221–1225.
- Adya, R. et al. (2012) Protective actions of globular and full-length adiponectin on human endothelial cells: novel insights into adiponectin-induced angiogenesis. *J. Vasc. Res.*, 49, 534–543.
- Jia, Z. et al. (2014) Adiponectin induces breast cancer cell migration and growth factor expression. *Cell Biochem. Biophys.*, 70, 1239–1245.
- Ouchi, N. et al. (2004) Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J. Biol. Chem.*, 279, 1304–1309.
- Tang, C.H. et al. (2009) Adiponectin increases motility of human prostate cancer cells via adipoR, p38, AMPK, and NF-kappaB pathways. *Prostate*, 69, 1781–1789.
- Cai, C. et al. (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. *Cancer Cell*, 20, 457–471.
- Gavin, J.R. 3rd et al. (1974) Insulin-dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture. *Proc. Natl Acad. Sci. USA.*, 71, 84–88.
- Madihe, A.M. et al. (2000) Differential expression of leptin receptor in high- and low-fat-fed Osborne-Mendel and SSB/Pl rats. *Obes. Res.*, 8, 467–474.
- Touvier, M. et al. (2013) Association between prediagnostic biomarkers of inflammation and endothelial function and cancer risk: a nested case-control study. *Am. J. Epidemiol.*, 177, 3–13.
- Stevens, V.L. et al. (2014) No association of plasma levels of adiponectin and c-peptide with risk of aggressive prostate cancer in the Cancer Prevention Study II Nutrition Cohort. *Cancer Epidemiol. Biomarkers Prev.*, 23, 890–892.
- Peters, K.E. et al. (2013) A comprehensive investigation of variants in genes encoding adiponectin (ADIPOQ) and its receptors (ADIPOR1/R2), and their association with serum adiponectin, type 2 diabetes, insulin resistance and the metabolic syndrome. *BMC Med. Genet.*, 14, 15.
- Mather, K.J. et al. (2012) Common variants in genes encoding adiponectin (ADIPOQ) and its receptors (ADIPOR1/2), adiponectin concentrations, and diabetes incidence in the Diabetes Prevention Program. *Diabet. Med.*, 29, 1579–1588.
- Civitaresse, A.E. et al. (2004) Adiponectin receptors gene expression and insulin sensitivity in non-diabetic Mexican Americans with or without a family history of Type 2 diabetes. *Diabetologia*, 47, 816–820.
- Yamauchi, T. et al. (2007) Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat. Med.*, 13, 332–339.
- Nakajima, A. et al. (2010) Molecular mechanisms linking adiponectin receptor signalling and cancer. *Open Obes. J.*, 2, 43–49.

-
46. Araneta, M.R. et al. (2007) Adiponectin and ghrelin levels and body size in normoglycemic Filipino, African-American, and white women. *Obesity (Silver Spring)*, 15, 2454–2462.
 47. Bush, N.C. et al. (2005) Adiponectin is lower among African Americans and is independently related to insulin sensitivity in children and adolescents. *Diabetes*, 54, 2772–2778.
 48. Considine, R.V. et al. (2008) Adiponectin and leptin in African Americans. *Obesity (Silver Spring)*, 16, 428–434.
 49. Okada-Iwabu, M. et al. (2013) A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. *Nature*, 503, 493–499.
 50. Holland, W.L. et al. (2013) Cell biology. Ronning after the adiponectin receptors. *Science*, 342, 1460–1461.