Protein expression of PTEN, insulin-like growth factor I receptor (IGF1R), and lethal prostate cancer: a prospective study

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ABSTRACT

Background: Loss of PTEN has been shown to be associated with aggressive behavior of prostate cancer. It is less clear that loss of PTEN also increases the risk of cancer mortality. We investigated the association between PTEN expression and prostate cancer mortality, and the potential effect modification by IGF1R, a direct activator of the PI3K pathway.

Methods: Protein expression in tumor were evaluated using tumor tissues obtained from 805 participants of the Physicians' Health and the Health Professionals Follow-up studies who were diagnosed with prostate cancer and underwent radical prostatectomy. Proportional hazard models were used to assess PTEN expression, and its interaction with IGF1R, in relation to lethal prostate cancer (cancer-specific death or distant metastases).

Results: Low PTEN expression was associated with an increase risk of lethal prostate cancer (HR = 1.7, 95% CI: 0.98-3.2, P for trend = 0.04). The association was attenuated after adjustment for Gleason grade, tumor stage, and PSA at diagnosis. A significant negative interaction between PTEN and IGF1R was found (P for interaction = 0.03). Either reduction in PTEN or increase in IGF1R expression was sufficient to worsen prognosis. Models including PTEN and IGF1R expression offer additional predicting power to prostate cancer survival, comparing to those only including demographic and clinical factors.

Conclusions: Low PTEN protein expression significantly increases the risk of lethal prostate cancer, particularly when the IGF1R expression remains at normal level.

Impact: PTEN and IGF1R expression in tumor are promising candidates for independent prognostic factors to predict lethal prostate cancer.

INTRODUCTION

The use of prostate-specific antigen (PSA) screening for prostate cancer has likely contributed to reductions in cancer-specific mortality (1, 2), while at the same time over-treatment is a concern for many PSA test-detected cancers (3, 4). The reason for such a controversy lies in the heterogeneous nature of prostate cancer, of which the clinical behaviors vary greatly (5-8). While many tumors grow slowly and would not pose a threat if left untreated, some clinically organ-confined tumors progress rapidly and develop metastasis even after local therapy. Therefore, it is of urgent need and great clinical importance to identify key factors or molecular events that can determine or predict the behaviors of the tumor.

One such marker for prostate cancer is phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor that negatively regulates the PI3K/Akt pathway (9, 10). Loss or inactivation of PTEN, usually leading to constitutive activation of the PI3K/Akt pathway, has been implicated in a number of human malignancies, including prostate cancer (11-13). In animal models, prostate-specific PTEN knockout can lead to tumorigenesis, progression, and metastasis of prostate cancer (14-16). In epidemiologic and clinical studies, loss or reduction of PTEN in prostate tumors has been shown to be associated with higher grade (17-20), more advanced stages (18, 20), greater angiogenesis (21), faster progression to androgen independence (22), and increased risk of recurrent disease (22-25). The role of PTEN loss in association with prostate cancer-specific mortality, however, is less clear. Both positive and null findings have been reported (18, 19, 26). Homozygous deletions or mutations of PTEN gene, resulting in total loss of PTEN activity, occur at relatively low frequencies of 10%-15% in primary prostate cancer (11, 21). However, a significantly higher proportion of tumors have reduced PTEN protein level (17, 20, 27), and such reductions may be sufficient to influence prostate cancer survival (24, 26).

One of the activators of the PI3K/Akt pathway is the insulin-like growth factor 1 (IGF1) (28, 29). Initiation of downstream signaling by IGF1 is predominantly mediated by the IGF1 receptor (IGF1R), which is ubiquitously expressed across different tissues, and promotes cell proliferation, differentiation, and inhibits apoptosis. Overexpression or constitutive activation of IGF1R is shown to promote tumor

formation in mouse models (30, 31). Several clinical studies reported high IGF1R expression has been detected in human tumor specimens and the expression level correlates with metastasis (32-34). Epidemiologic studies on IGF1 signaling in relation to risk of prostate cancer have nearly exclusively focused on circulating IGF1 and IGF binding proteins (35-46). Although some recent findings have been null, the majority of the studies support a positive association between IGF1 and prostate cancer risk.

We hypothesize that reduction or loss of PTEN expression in prostate tumor contributes to cancer progression. Since the PI3K/PTEN/Akt pathway is downstream of IGF1/IGF1R, the effect of PTEN loss may be modified by IGF1 signaling. To test our hypotheses, we examined tumor protein expression of PTEN and IGF1R from specimens of 805 men who were diagnosed with prostate cancer and underwent radical prostatectomy as therapy, and followed them prospectively for cancer-specific mortality or development of distant metastasis. We evaluated whether PTEN protein expression in tumor is related to the risk of lethal prostate cancer, defined by cancer-specific mortality or distant metastasis. We also investigated IGF1R expression in tumor as a potential effect modifier for the association between PTEN expression and lethal prostate cancer risk. The research was approved by the Institutional Review Board at the Harvard School of Public Health and the Partners Health Care.

MATERIALS AND METHODS

Study Population

The men with prostate cancer in this study were participants in the prospective Physicians' Health Study (PHS) and Health Professionals Follow-up Study (HPFS). The PHS was a randomized trial among 29,067 US male physicians ages 40-84 at randomization in the primary prevention of cancer and cardiovascular disease. Participants are sent annual questionnaires to ascertain disease endpoints as well as lifestyle, dietary and medical covariate data as part of the main trial. The HPFS is an ongoing prospective cohort study of 51,529 U.S. male health professionals who were aged 40 to

75 years at baseline in 1986. The participants have been sent a questionnaire every 2 years since 1986 to update their information on lifestyle factors, medical history, and disease outcomes.

The PHS and HPFS Prostate Tumor Cohort is a sample of participants for whom we had retrieved archival formalin-fixed, paraffin embedded prostatectomy (95%) and TURP (5%) tumor specimens from these men. The current analysis is based among 804 men (329 from PHS and 475 from HPFS) for whom the first batch of molecular assessment of PTEN expression was completed.

Ascertainment of Outcome

In both the PHS and HPFS cohorts, cases of prostate cancer were identified by self-report, then confirmed by review of medical records and pathology reports. Clinical information, such as tumor stage and PSA at diagnosis, was acquired through a standardized review of medical records. The cohorts were followed prospectively for cancer and all-cause mortality. Deaths were ascertained through repeated mailings, telephone calls to non-respondents, and searches of the National Death Index. All causes of death were confirmed by extensive review of death certificates and medical records. Follow-up for cancer was >96% complete and for mortality >98% complete. Participants who had prostate cancer were separately followed. Detailed information on treatment and development of metastasis was obtained via additional questionnaires and collection of medical records.

Immunohistochemistry

Angiogenesis. Protein expression of endothelial cell marker CD34 was ascertained on 5 micron sections using the *anti-CD34 mouse monoclonal antibody* (QBE ND 10, BioGenex, CA, diluted 1:200) and peroxidase blocking reagent (Dual Endogenous Enzyme Block, DakoCytomation, CA). Detection was accomplished by a peroxidase labeled polymer conjugated to anti-mouse IgG (DakoCytomation, CA), liquid 3,3'-diaminobenzidine (DAB)+ Substrate Chromogen System (K3468), and DakoCytomation EnVision+Dual Link System (DakoCytomation, CA). Immunohistochemistry was performed in an OptiMax automated cell Staining System (BioGenex Laboratories, CA). Slides were counterstained with hematoxylin (Sigma-Aldrich, MO). The size and architecture of microvessels were quantified by Semi-automated image analysis using Image ProPlus 4.5 software (Media Cybernetics, MD). Vessel size was determined as the average area comprised by a vessel (μm^2). The irregularity

of the vessel lumen was calculated by perimeter^2 / 4*Π*area, with a value of 1.0 indicating a perfect circle and values greater than 1.0 indicating increasing irregularity.

Tissue Microarrays. To increase efficiency of immunohistochemical staining, we constructed high-density tumor tissue microarrays (TMA). Our pathologists (ML, MF, SF, RF) reviewed hematoxylin and eosin slides to confirm prostate cancer, and to provide uniform Gleason grading (47). TMAs were constructed by sampling 0.6 mm paraffin embedded tissue cores (at least 3 cores per subject) and embedding the cores on a recipient array block.

PTEN. Cytoplasmic expression of PTEN in tumor cells was ascertained using the *Anti-PTEN rabbit polyclonal antibody* (Zymed, San Francisco, CA; diluted 1:200) and was quantified using automated image analysis. Briefly, 4 micron sections of each TMA were mounted on a glass slide, deparaffinized and microwaved for antigen retrieval in citrate-based buffer. Hematoxylin was used as a counterstain and diaminobenzadine (DAB) for the immunohistochemical staining. The stained slides were then scanned using the Ariol Pathology and Imaging System (Applied Imaging, San Jose, CA), and PTEN expression was quantified using the Ariol Immunohistochemical Module. Following image acquisition of the stained slide, a custom mask was made for each core to limit analysis to tumor cells. The IHC module in Ariol outputs information on the staining intensity for a given core (range 0-255, mean and mode intensity across core), the area of DAB staining and blue counter-stain which allows calculation of percent of tumor tissue staining positive for PTEN (0-100%).

IGF1R and IR. Immunohistochemical staining was conducted on 5 micron sections of each TMA using the *Anti-Insulin Receptor, B subunit*, Rabbit immunoaffinity purified IgG (Upstate Cell Signaling Solutions, Lake Placid, NY) and the *IGF-1R beta R rabbit polyclonal antibody* (Santa Cruz Biotechnology Inc, Santa Cruz, CA) with enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit (Ventana autostainer model Discover XT™, Ventana Medical System, Tuscan, AZ). Nonspecific reactivity was assessed by omission of the primary antibody. The specificity of staining for IR was confirmed by using placenta as a positive control. The slide was scanned with the BLISS system (Bacus Lab, North Lombard, IL) and scored manually by two pathologists who were blinded to clinical outcomes. For both receptors, intensity was scored from 0 to +3 – 0 (no staining by

any tumor cells, 1 (faint or focal staining), 2 (moderate intensity in a convincing number of cells), and 3 (intense staining by a sufficient number of cells).

Apoptosis. The TUNEL assay was used on 5 micron sections to identify the percentage of tumor cells undergoing apoptosis. The procedure was carried out using the Apoptag Peroxidase In situ kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The entire area of each tumor core was evaluated. Apoptosis was quantified as the percentage of positively-stained area over the whole tumor area.

Ki-67. The expression of Ki-67, a cell proliferation marker, was assessed on 5 micron sections using a rabbit polyclonal antibody (Vector Labs, Burlingame, CA; diluted 1:1,500). After immunohistochemical staining, the tumor areas of each core were selected for quantitative image analysis using the Ariol instrument SL-50 (Applied Imaging, San Jose, CA). Cell proliferation was quantified as the percentage of Ki-67-positive nuclei over all tumor nuclei.

Statistical Analysis

In the analyses presented here, we used the baseline questionnaire data when the participants entered the original HPFS and PHS studies as baseline for demographic measures.

For each individual, we calculated the minimum, mean, and maximum values for percent area staining and staining intensity of PTEN across the TMA cores for an individual. These values were qualitatively similar; therefore we only presented the analyses using the mean values. We consider the percent area staining, reflecting the percent of tumor tissue with PTEN expression, as the most relevant measure to evaluate the potential role of PTEN in cancer progression. Additionally, we also generated a combined staining score, by multiplying the mean percent staining and the mean staining intensity, to reflect total PTEN expression in the tumor. These two measures were used as continuous variables in the analyses. They were also divided into quartiles according to the cohort distribution. In addition, we created an arbitrary cutoff point for each of these two measures by combining the bottom three quartiles as the low expression group. Less than 32% is considered as low percent staining, and less than 32.25 as low score.

We compared clinical characteristics of prostate cancer cases including age at diagnosis, baseline body mass index (BMI), tumor stage, Gleason grade, and PSA at diagnosis, as well as cellular biomarkers for characteristics of angiogenesis (microvessel size and irregularity), apoptosis, and cell proliferation, across quartiles of PTEN expression. We calculated a p-for-trend value from ANOVA for age at diagnosis, baseline BMI, angiogenic characteristics, apoptosis, and cell proliferation across PTEN quartiles. Using the Mantel-Haenszel statistic, we tested whether there is a linear trend for tumor stage, Gleason grade, or PSA at diagnosis across quartiles of PTEN expression.

We employed time-to-event analyses to evaluate the association of PTEN expression on development of lethal prostate cancer (cancer-specific death or development of distant metastasis). Cases were diagnosed with prostate cancer between 1983-2005 in PHS and 1986-2002 in HPFS. Person-time for each individual was calculated from the date of cancer diagnosis to development of metastases, death or end of follow-up (July 1, 2010 for the HPFS and March 1, 2010 for the PHS). Hazard ratios (HR) and 95% confidence intervals (CI) were estimated from the Cox proportional hazard regression models. We adjusted for age at diagnosis (5-year categories), diagnosis era (pre-PSA era: before 1989, peri-PSA era: 1989-1993, PSA era: after 1993), baseline BMI (kg/m^2, continuous), and baseline smoking status (current, non-current). In additional models, we examined PTEN expression and lethal prostate cancer independent of tumor stage (T1/T2, T3, T4), Gleason score (6 or less, 7-10), and PSA at diagnosis (ng/ml; <4.1, 4.1-9.9, 10 or higher, or missing). The trend tests across quartiles of percent staining and the multiplicative score were performed by assigning median values of these categories and entering these values as continuous terms in the Cox model.

To investigate whether the associations between PTEN expression and lethal prostate cancer risk differ between the HPFS and PHS cohorts, we conducted survival analyses in the HPFS and PHS separately, and calculated the Q statistics, which follows an approximate $\chi 2$ distribution with 1 degree of freedom. Due to the absence of heterogeneity, the two cohorts were merged for all analyses.

We assessed whether the association between of PTEN expression and the development of lethal prostate cancer differed by IGF1R expression in a subset of individuals (N=651), who have both

the PTEN and IGF1R expression measured. IGF1R expression was measured by a multiplicative score, similar to that of PTEN expression. The top tertile of the IGF1R multiplicative score was arbitrarily defined as high IGF1R expression. We performed stratified survival analyses by the IGF1R multiplicative score to evaluate the associations of percent PTEN staining and PTEN multiplicative staining score and lethal prostate cancer risk. We also generated interaction terms between PTEN expression measures and the IGF1R multiplicative score, and fitted multivariate Cox models with main effect measures and covariates. The significance of the interaction was determined using Wald test to test the beta-coefficients of the cross-product terms.

We evaluated the ability of PTEN and IGF1R expression to predict 10-year survival after diagnosis of prostate cancer using logistic regression, comparing the base model containing only clinical and demographic covariates to the model also containing PTEN and IGF1R expression and their interaction. Area under curve (AUC), net reclassification improvement (NRI), and integrated discrimination index (IDI) were calculated for these two nested models.

All statistical analyses were two-sided and a P-value of less than 0.05 was considered statistically significant. We conducted all analyses using the SAS software (SAS Institute, Inc., Version 9.1, Cary, NC).

RESULTS

The baseline characteristics of the HPFS and PHS Tumor Cohorts are presented in **Table 1**. These two cohorts were demographically and clinically similar. In HPFS, 475 men who were diagnosed with prostate cancer between 1986 and 2002 have been followed prospectively for clinical progression and mortality, with a median follow-up of 13.2 years. The average age at cancer diagnosis was 65.6 years. A total of 38 prostate cancer-specific death and 10 cases of distant metastatic prostate cancer were documented. In PHS, 330 men were diagnosed with prostate cancer between 1983-2005. The mean age at diagnosis was 66.4 years. During a median follow-up of 10.9 years, 25 cancer death and 3 distant metastases occurred. For both cohorts, the majority of the cancers were diagnosed after the clinical introduction of PSA test as prostate cancer screening. The

proportion of current smokers was relatively low at baseline in both cohorts, compared to the general population.

The characteristics of prostate cancer cases were compared across quartiles of PTEN expression, as shown in **Table 2**. No significant differences in age at cancer diagnosis, baseline BMI, or PSA at diagnosis were observed across quartiles of percent area for PTEN staining. Tumors with lower percent area for PTEN staining were more likely to be advanced stage (P for trend = 0.06) and higher Gleason grade (P for trend = 0.049). The results were similar when PTEN expression was stratified according to the multiplicative staining score (data not shown). Higher expression of PTEN appeared to be associated with more apoptosis in tumor cells (P for trend = 0.002). Such a trend was not present for either characteristics of angiogenesis including microvessel size and irregularity or cell proliferation.

We evaluated the association between PTEN expression in tumor and lethal prostate cancer (cancer death/distant metastasis); the results are shown in **Table 3**. Using the percent of tumor tissue with positive staining as a continuous variable, every 10% decrease in staining conferred a 20% increase in risk of lethal disease, after adjusting for age at diagnosis, diagnosis era, baseline BMI and smoking status. Men with the lowest percent staining (bottom quartile) were at markedly higher risk for lethal cancer (HR = 2.4, 95% CI: 1.2-4.7), compared to those with the highest percent staining (top quartile) (p for trend = 0.04). Low percent of tumor tissue stained positive for PTEN protein (< 32%) was associated with a borderline significant HR of 1.7 (95% CI: 0.98-3.2). However, when we further adjusted for clinical characteristics of the tumor, such as tumor stage, Gleason grade and PSA at diagnosis, percent of tumor tissue with PTEN expression no longer predicted lethal prostate cancer. The multiplicative score for PTEN staining, as a measure for total PTEN protein in tumor, yielded very similar findings.

To examine the potential interaction between PTEN and IGF1R expression, we stratified the study population by IGF1R staining score. As shown in **Table 4**, the associations between measures of PTEN expression and development of lethal prostate cancer varied according to the level of IGF1R expression. When the expression of IGF1R protein is low in the tumor, low PTEN expression was

significantly predictive of lethal disease, adjusted for both demographic and clinical parameters. A statistically significant trend in risk of lethal prostate cancer was present across the quartiles of PTEN expression (P = 0.01 for percent and P = 0.04 for score). Low percent and low score for PTEN staining were both associated a more-than-10-fold increase in the multivariate HR. However, when the expression of IGF1R protein is high in the tumor, no association between measures of PTEN expression and lethal prostate cancer was observed. A strong negative interaction was observed between PTEN and IGF1R expression (P = 0.03). In additional multivariate survival analyses presented in **Figure 1**, we used men with low score for IGF1R staining and high percent of tumor with PTEN staining as the reference group. High score for IGF1R staining alone was associated with an HR of 13.8 (95% CI: 1.7-112.8). Similarly, low percent of tumor with PTEN staining alone was associated with an HR of 11.4 (95% CI: 1.5-84.3). However, combining these two conditions did not confer additional risk for lethal prostate cancer (HR = 12.0, 95% CI: 1.6-90.2).

To assess the ability of PTEN and IGF1R expression to predict 10-year survival after diagnosis of prostate cancer, we compared the base logistic regression model that included demographic factors such as smoking status and BMI, as well as prognostic factors including patient age, tumor stage, Gleason score, and PSA at diagnosis, to the model also including percent for PTEN staining, score for IGF1R staining, and their interaction. Adding PTEN and IGF1R expression to the base model increased AUC from 0.837 to 0.864, and improved NRI (NRI = 0.191, p = 0.004) and IDI (IDI = 0.0129, p = 0.0007) significantly. These results suggest that inclusion of PTEN and IGF1R expression and their interaction in the base model offer further predicting power for prostate cancer prognosis.

DISCUSSION

To our knowledge, our study is one of the largest studies to evaluate the role of PTEN protein expression level in relation to the risk of lethal prostate cancer. We observed a significant positive association between reduction in PTEN protein expression and development of lethal disease, although the association was attenuated after adjustment for tumor stage, Gleason grade, and PSA

levels. We are also the first to report that the association of PTEN tumor expression with clinical outcome is strongly modified by the level of IGF1R expression in the tumor. These two factors negatively interact with each other: Unfavorable changes in protein expression of either biomarker alone are associated with increased risk, but combined unfavorable changes in both do not confer additional harm. PTEN and IGF1R expression in tumor predict prostate cancer survival independently of clinical prognostic factors.

Loss of PTEN expression has been shown to be correlated with indicators of poorer prognosis, such as high grade (17-20), advanced stage (18, 20), and the number of microvessels, a marker of angiogenesis (21). Several studies also reported that PTEN inactivation is associated with biochemical or clinical recurrence of prostate cancer (22-25). Three recent studies examined the effect of PTEN loss in relation to cause-specific death, which is the most relevant outcome for prostate cancer (18, 19, 26). Reid et al. performed fluorescence in situ hybridization (FISH) assays in TMAs to detect PTEN loss in 308 patients with conservatively managed prostate cancer and reported that loss of PTEN alone was not associated with prostate cancer survival (18). Pourmand et al. used tissue microdissection and polymerase chain reaction/single-strand conformation polymorphism methods to analyze *PTEN* mutations in 51 prostate tumor specimens obtained from radical prostatectomy or transurethral resection (19). Patients with *PTEN* mutations were found to have lower survival rates ($P = 0.001$). When Cox regression models were employed, with adjustment for Gleason grade, age and PSA at diagnosis, *PTEN* mutations were no longer associated with death by prostate cancer ($P = 0.60$). In the study by Sircar et al. (26), TMAs were constructed from transurethral resection samples of 59 patients with hormone refractory prostate cancer. The four-color FISH strategy was employed to evaluate genomic deletion of *PTEN*. The number of *PTEN* gene deletions (undeleted, heterozygous, and homozygous) was significantly correlated with prostate cancer-specific mortality ($r = 0.332$, $P = 0.04$). Yet presence versus absence of any *PTEN* deletion, by itself, was not correlated with cancer death ($r = 0.189$, $P = 0.3$). These findings also imply that the change in *PTEN* protein level is more relevant in association with prostate cancer survival.

The role of circulating IGF1 and its binding proteins such as IGFBP3 in prostate cancer has been extensively investigated (35-46). Evidence that high levels of circulating IGF1 significantly increase the risk of total prostate cancer became less than convincing with some recent reports of null findings (37, 41, 43). However, several studies, including the PHS and HPFS, have shown that IGF1 is more strongly associated with low-grade prostate cancer (35, 40, 45). A recent pooled analysis of individual patient data from 12 prospective studies yielded a significantly elevated risk for prostate cancer, comparing the highest vs. the lowest quintiles of the serum IGF-1 concentration (46). This increased risk became more pronounced when the analysis was restricted to low-grade prostate tumors, suggesting poorly-differentiated tumors, which are more autonomous, are less sensitive to the stimulus of IGF1. Such reduced dependence of IGF1 may be the result of alterations in expression of the downstream signaling pathway molecules, including those in the PI3K/Akt pathway. As described previously, loss of PTEN is strongly associated with high grade prostate tumor. We also found that lower PTEN levels were significantly associated with higher Gleason grade. Thus our finding that higher levels of IGF1R expression in tumor was related to lethal prostate cancer when PTEN level was high whereas lower PTEN was related to lethal outcome regardless IGF1R further support the notion that poorly-differentiated tumors are less sensitive to IGF1/IGF1R.

In addition to IGF1R, IGF1 also binds to and activate insulin receptor (IR) at a weaker affinity (29). However, no interaction between PTEN and IR expression was present in our study population (data not shown), suggesting the interaction between PTEN and IGF1R is specific to the IGF1/PI3K/Akt signaling cascade and the involvement of insulin signaling may be limited.

There are potential limitations in our study to be considered. First, the PHS and HPFS Tumor Cohort consists of physicians and health professionals, are mainly Caucasian, and have relatively high socio-economic status. Thus it is not a random sample of the general population. This may limit the generalizability of the results, though the validity of the study is not undermined. We may be able to gain insights into the underlying biological mechanism through which reduced PTEN expression may promote prostate cancer progression. Secondly, we have relatively few deaths or distant metastases from prostate cancer considering our large sample size. However, despite the small

number of outcomes, we had sufficient power to detect the interaction between PTEN and IGF1R expression. Thirdly, the tumor specimens were obtained only when men underwent prostatectomy or TURP as curative treatment for their cancer. These are largely localized, organ-confined tumors. Those who were diagnosed with more advanced cancers may be ineligible for surgical intervention and have undergone hormonal, radiation therapy, or chemotherapy. Since loss or reduction of PTEN is generally regarded as a late event in prostatic tumorigenesis, and is associated with more malignant tumors and worse prognosis, it is likely that our analysis underestimates the role of PTEN expression in relation to prostate cancer mortality. The use of tumor blocks may generate certain heterogeneity for these tumors were diagnosed at different times, and fixed and processed in different pathological labs. Last, the presence of missing data such as PSA at diagnosis and baseline smoking status can lead to residual confounding by these factors. Uncontrolled confounding by unknown or unmeasured factors can not be ruled out either.

The prospective study design with long-term and complete follow-up adds to the strength of our analysis. Clinical characteristics of the cancers were systematically extracted from medical records in a standardized format. The study pathologists reviewed the records and provided uniform Gleason grades for all cases. These approaches should contribute to reduction in measurement errors. Additionally, we used cause-specific death and distant metastasis as the outcome, which are the most clinically relevant endpoints for prostate cancer.

In conclusion, low levels of PTEN protein are associated with an increased risk of lethal prostate cancer. This association is strongly modified by IGF1R expression in the tumor. Our results reinforce the idea that the PI3K/PTEN/Akt pathway plays a critical role in prostate cancer survival. PTEN and IGF1R are eligible independent predictors for prognosis. Their interaction may shine light on the complex picture of IGF axis in prostate tumorigenesis and cancer progression.

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TABLES

Table 1 Clinical and demographic characteristics of the HPFS (1986-2010) and PHS (1983-2010) Prostate Tumor Cohort

	HPFS (N=475)	PHS (N=330)
Continuous		
Age at baseline (yr), mean (sd)	57.7 (7.4)	56.3 (7.6)
Age at diagnosis (yr), mean (sd)	65.6 (6.3)	66.4 (6.2)
Follow-up (month), median (range)	158.0 (1.0-281.0)	130.9 (1.0-277.8)
Baseline BMI (kg/m^2), median (range)	24.8 (18.6-61.5)	24.4 (18.3-35.9)
Mean percent area for PTEN+, mean (sd)	0.25 (0.22)	0.20 (0.18)
Mean multiplicative score for PTEN+, mean (sd)	26.0 (24.4)	19.9 (20.1)
Categorical	N (%)	N (%)
Outcomes		
Prostate cancer death	38 (8.0)	25 (7.6)
Distant metastasis	10 (2.1)	3 (0.9)
Tumor stage		
pT1b-pT2N0M0	323 (68.0)	254 (77.0)
pT3N0M0	132 (27.8)	63 (19.1)
pT4/N1/M1	20 (4.2)	13 (3.9)
Gleason grade		
2-6	95 (20.0)	88 (26.8)
7 (3+4)	116 (24.4)	67 (20.3)
7 (4+3)	190 (40.0)	120 (36.4)
8-10	74 (15.6)	54 (16.4) *(Missing 1 record)*
Smoking status		
Never	238 (50.1)	164 (49.7)
Past	191 (40.2)	120 (36.4)
Current	46 (9.7)	24 (7.3)
Missing	0 (0)	22 (6.7)
PSA at diagnosis		
< 4	48 (10.1)	31 (9.4)
4-10	214 (45.1)	184 (55.8)
>10	136 (28.6)	67 (20.3)
Missing	77 (16.2)	48 (14.6)
Year of diagnosis		
Before 1990 (Pre-PSA era)	35 (7.4)	25 (7.6)
1990-1993 (Peri-PSA era)	155 (32.6)	63 (19.1)
After 1993 (PSA era)	285 (60.0)	242 (73.3)

Table 2 Characteristics of prostate cancer cases across quartiles of PTEN expression in the HPFS (1986-2010) and PHS (1983-2010)

Prostate Tumor Cohort

	N[§]	Overall	Mean percent area of PTEN staining				P for Trend
			Q1	Q2	Q3	Q4	
Mean percent area of PTEN+, mean (sd)	805	0.23 (0.21)	0.04 (0.02)	0.11 (0.03)	0.23 (0.05)	0.53 (0.17)	
Age at diagnosis (yr), mean (sd)	805	65.9 (6.3)	65.9 (6.4)	65.3 (6.1)	66.2 (6.2)	66.5 (6.3)	0.2[†]
Baseline BMI (kg/m^2), mean (sd)	805	24.9 (3.0)	24.8 (2.6)	25.0 (3.9)	24.9 (2.8)	24.9 (2.4)	0.8[†]
Tumor stage, N (%)	805						
pT1b-pT2N0M0		577 (71.7)	128 (67.0)	143 (68.4)	154 (76.6)	152 (74.5)	
pT3N0M0		195 (24.7)	54 (28.3)	57 (27.3)	41 (20.4)	43 (21.1)	
pT4/N1/M1		33 (4.1)	9 (4.7)	9 (4.3)	6 (3.0)	9 (4.4)	0.06[‡]
Gleason grade, N (%)	804						
2-6		183 (22.8)	36 (19.0)	46 (22.0)	45 (22.4)	56 (27.5)	
7		493 (61.3)	117 (61.6)	131 (62.7)	129 (64.2)	116 (56.9)	
8-10		128 (15.9)	37 (19.5)	32 (15.3)	27 (13.4)	32 (15.7)	0.049[‡]
PSA at diagnosis, N (%)	680						
< 4		79 (11.6)	18 (11.1)	16 (9.0)	24 (14.6)	21 (11.9)	
4-10		398 (58.5)	94 (58.0)	110 (62.2)	92 (56.1)	102 (57.6)	
>10		203 (29.9)	50 (30.9)	51 (28.8)	48 (29.3)	54 (30.5)	0.7[‡]
Angiogenesis - CD34 staining	351						
Vessel area[‖] (μm^2), mean (sd)		549.2 (265.6)	502.0 (246.9)	556.4 (248.6)	596.4 (319.7)	530.4 (233.0)	0.3[†]
Irregularity of vessel lumen[¶], mean (sd)		3.87 (1.00)	3.88 (0.99)	3.67 (0.93)	3.89 (1.06)	4.01 (1.00)	0.3[†]
Apoptosis - TUNEL assay							
Percentage of stained area, mean (sd)	669	2.2 (4.5)	1.6 (3.6)	2.1 (4.2)	1.8 (3.4)	3.3 (6.3)	0.002[†]
Cell proliferation - Ki-67 staining							
Percentage of positive nuclei, mean (sd)	773	0.73 (2.6)	0.81 (2.8)	0.48 (0.99)	0.59 (1.4)	1.04 (3.9)	0.07[†]

§ Missing data if N is not equal to 805

‖ Smaller vessels are more angiogenic

¶ Irregular vessels are more angiogenic, a score of 1.0 indicates a perfect circle

† P for trend was calculated using ANOVA

‡ P for trend was calculated from the Mantel-Haenszel statistic

Table 3 Hazard ratios (HR) and 95% Confidence Interval (CI) of PTEN expression to predict time to lethal prostate cancer* in the HPFS (1986-2010) and PHS (1983-2010) Prostate Tumor Cohort

PTEN expression	No. of lethal cases	HR† (95% CI)	HR‡ (95% CI)	HR§ (95% CI)
Percent Staining				
Per 10% decrease	76	1.2 (1.0, 1.3)	1.2 (1.0, 1.3)	1.1 (0.93, 1.2)
Q1 - Lower	24	2.4 (1.2, 4.7)	2.4 (1.2, 4.7)	1.6 (0.82, 3.3)
Q2	15	1.2 (0.59, 2.7)	1.2 (0.56, 2.6)	1.0 (0.44, 2.1)
Q3	22	1.7 (0.87, 3.5)	1.7 (0.87, 3.5)	1.6 (0.77, 3.2)
Q4 - Higher	15	1	1	1
Test for trend		P = 0.04	P = 0.04	P = 0.33
Low percent (<32%)	61	1.8 (0.98, 3.2)	1.7 (0.98, 3.2)	1.4 (0.76, 2.6)
Multiplicative score				
Per SD decrease	76	1.4 (1.1, 1.8)	1.4 (1.1, 1.8)	1.2 (0.88, 1.6)
Q1 - Lower	24	2.6 (1.3, 5.2)	2.6 (1.3, 5.2)	1.8 (0.85, 3.6)
Q2	14	1.4 (0.62, 3.0)	1.3 (0.6, 3.0)	1.1 (0.48, 2.5)
Q3	25	2.3 (1.1, 4.7)	2.3 (1.1, 4.7)	2.0 (0.97, 4.3)
Q4 - Higher	13	1	1	1
Test for trend		P = 0.03	P = 0.03	P = 0.27
Low score (<32.25)	63	2.1 (1.1, 3.9)	2.1 (1.1, 3.9)	1.6 (0.86, 3.1)

* Lethal prostate cancer is defined as fatal prostate cancer or distant metastasis

† Hazard ratios adjusted for age at diagnosis and diagnosis era

‡ Hazard ratios adjusted for age at diagnosis, diagnosis era, baseline BMI and smoking status

§ Hazard ratios adjusted for age at diagnosis, diagnosis era, baseline BMI, smoking status, Gleason grade, and PSA at diagnosis

Table 4 HR and 95% CI of PTEN expression to predict time to lethal prostate cancer* in the HPFS (1986-2010) and PHS (1983-2010) Prostate Tumor Cohort, stratified by IGF1R expression

PTEN expression	Low score of IGF1R expression		High score of IGF1R expression		$P_{for\ interaction}$
	No. of lethal cases	HR† (95% CI)	No. of lethal cases	HR† (95% CI)	
Percent Staining					
Per 10% decrease	31	1.3 (0.96, 1.7)	33	0.96 (0.77, 1.2)	0.14‡
Q1 - Lower	12	17.6 (1.7, 184)	10	0.77 (0.26, 2.2)	
Q2	5	10.4 (0.94, 115)	6	0.49 (0.14, 1.8)	
Q3	12	13.3 (1.3, 134)	9	1.1 (0.37, 3.6)	
Q4 - Higher	2	1	8	1	
Test for trend		P = 0.01		P = 0.44	
Low percent (<32%)	29	13.8 (1.5, 130)	25	0.78 (0.31, 2.0)	0.03‡
Multiplicative score					
Per SD decrease	31	1.7 (0.92, 3.2)	33	0.94 (0.60, 1.5)	0.15‡
Q1 - Lower	12	12.7 (1.3, 127)	10	0.98 (0.30, 3.1)	
Q2	4	7.6 (0.66, 87)	6	0.78 (0.20, 3.1)	
Q3	13	12.4 (1.2, 132)	11	1.9 (0.57, 6.3)	
Q4 - Higher	2	1	6	1	
Test for trend		P = 0.04		P = 0.77	
Low score (<32.25)	29	11.3 (1.2, 109)	27	1.2 (0.41, 3.6)	0.09‡

* Lethal prostate cancer is defined as fatal prostate cancer or distant metastasis

† Hazard ratios adjusted for age at diagnosis, diagnosis era, baseline BMI, smoking status, Gleason grade, and PSA at diagnosis

‡ Wald test was used to test the beta-coefficients of the cross-product terms between PTEN and IGF1R expression

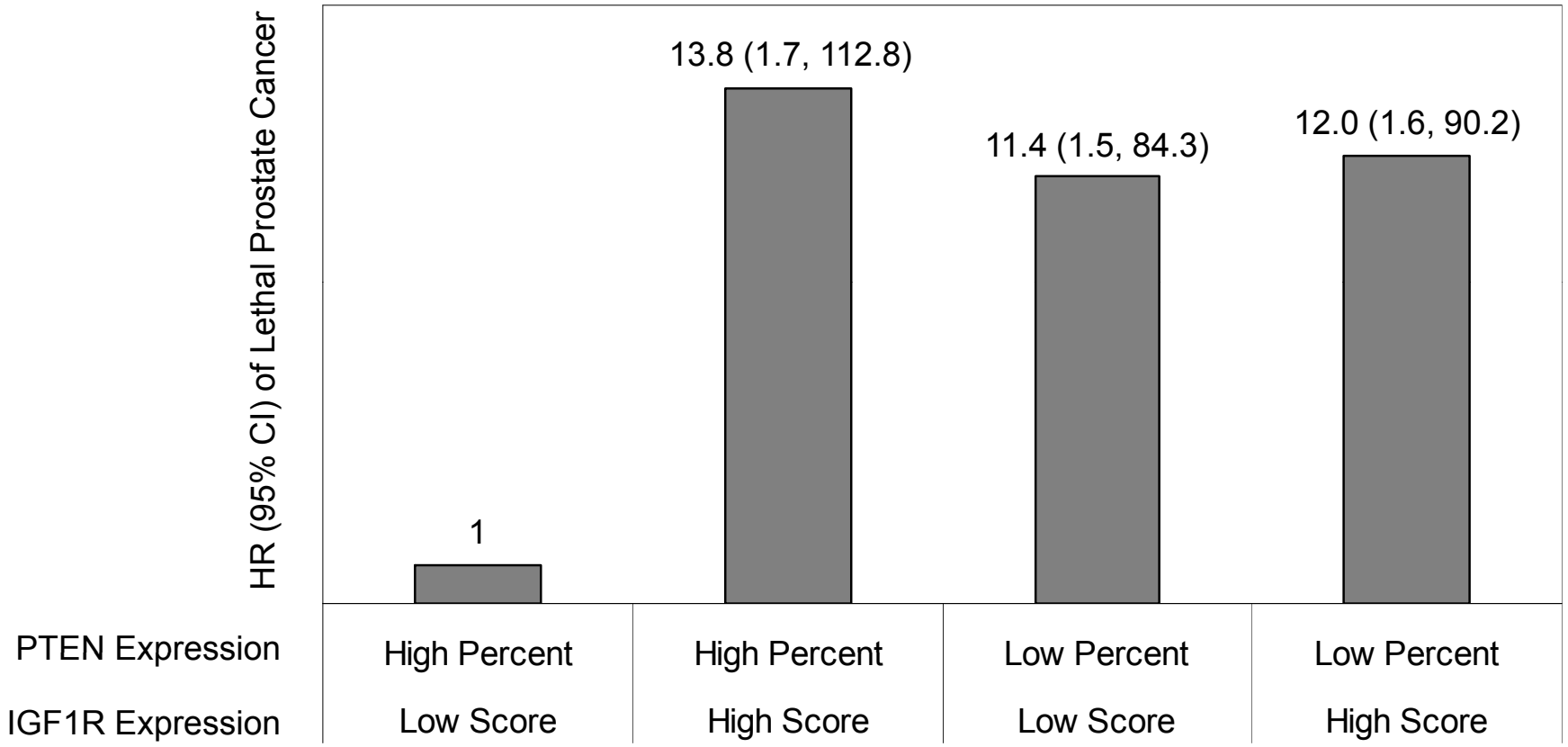
FIGURE LEGENDS

Figure 1. HR[†] and 95% CI of PTEN and IGF1R expression to predict time to lethal prostate cancer* in the HPFS (1986-2010) and PHS (1983-2010) Prostate Tumor Cohort

* Lethal prostate cancer is defined as fatal prostate cancer or distant metastasis.

[†] Hazard ratios adjusted for age at diagnosis, diagnosis era, baseline BMI, smoking status, Gleason grade, and PSA at diagnosis

Figure 1



† Hazard ratios adjusted for age at diagnosis, diagnosis era, baseline BMI, smoking status, Gleason grade, and PSA at diagnosis