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Stress-Related Signaling Pathways in Lethal and Non-Lethal Prostate Cancer

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Abstract

Purpose—Recent data suggest that neuroendocrine signaling may influence progression in some cancers. We aimed to determine whether genes within the five major stress-related signaling pathways are differentially expressed in tumor tissue when comparing prostate cancer patients with lethal and non-lethal disease.

Experimental Design—We measured mRNA expression of 51 selected genes involved in predetermined stress-related signaling pathways (adrenergic, glucocorticoid, dopaminergic, serotoninergic, and muscarinic systems) in tumor tissue and normal prostate tissue collected from prostate cancer patients in the Physicians' Health Study (n=150; n=82 with normal) and the Health Professionals Follow-Up Study (n=254; n=120 with normal). We assessed differences in pathway expression in relation to prostate cancer lethality as the primary outcome, and to biomarkers as secondary outcomes.

Results—Differential mRNA expression of genes within the adrenergic (p=0.001), glucocorticoid (p<0.0001), serotoninergic (p=0.0019), and muscarinic (p=0.0045) pathways in tumor tissue was associated with the risk of lethality. The adrenergic pathway was also statistically significant (p=0.001) when comparing against differential expression of genes not involved in the pathways. In adjacent normal prostate tissue, none of the pathways was clearly differentially expressed between lethal and non-lethal prostate cancer. The glucocorticoid and adrenergic pathways were associated with cell proliferation, while the glucocorticoid pathway was additionally associated with angiogenesis and perineural invasion.

Conclusions—Our study suggests that stress-related signaling pathways, particularly the adrenergic and glucocorticoid, may be dysregulated in the tumors of men whose prostate cancer proves to be lethal, and motivates further investigation of these pathways in functional studies.

Keywords

stress; signaling pathways; prostate cancer; survival

Introduction

A novel hypothesis stating that stress may influence cancer progression through neuroendocrine pathways has recently been proposed (1). The underpinnings include evidence that stress-induced neurotransmitters and hormones, including dopamine (2), serotonin (3), epinephrine (4), norepinephrine (4), acetylcholine (5), and glucocorticoids (6) can alter tumor microenvironment and impact tumor progression through influence on immune cells, cancer cells, and angiogenesis in a variety of cancers.

The hypothesis is of specific relevance for prostate cancer, where experiments in cell lines and animal models have shown that β2-adrenergic receptor (ADRB2) activation inhibits apoptosis and stimulates cell migration (7,8). Although debated (9), results from two large

observational studies suggested that use of β–adrenergic receptor blocking agents may improve cancer-specific survival among prostate cancer patients (10,11). The role of the autonomous nervous system in prostate cancer progression has been further demonstrated in mouse models: while activation of the adrenergic receptor was involved primarily in the early phase of prostate cancer development, muscarinic receptor activation promoted dissemination (5).

The glucocorticoid signaling system represents another stress-activated system of potential relevance to tumor progression. Although glucocorticoids are recognized to benefit hormone-refractory prostate cancer patients as pituitary suppressants of androgen production (12), they have also been reported to lend treatment resistance to prostate cancer cells and to suppress immune function (13,14). Other potentially relevant stress pathways include the serotoninergic and dopaminergic signaling systems. For example, subtypes 1A and 1B of the serotoninergic receptor are overexpressed in prostate cancer tissue, especially in high-grade tumors (15). Dopamine secretion on the other hand may hamper prostate cancer progression by normalizing the structure of aberrant tumor blood vessels (16).

However, associations of stress-related signaling pathways with lethal prostate cancer have not been extensively investigated in human subjects (17). We therefore undertook an integrative molecular study within a large cohort of US prostate cancer patients to test the hypothesis that mRNA expression of genes within five major stress-related signaling pathways are differentially expressed in the tumor tissue of men with lethal prostate cancer compared to men with non-lethal disease. Moreover, we assessed differences in mRNA expression with regard to clinical characteristics such as Gleason grade, tumor stage, and tumor characteristics including the extent of cell proliferation, apoptosis, angiogenesis, and perineural invasion.

Patients and Methods

Study Populations

The study was nested among US men with histologically confirmed prostate cancer who were participants in the Physicians' Health Study (PHS) (18) and the Health Professionals Follow-up Study (HPFS) (19). Written informed consent was obtained from each subject.

The PHS and HPFS Prostate Tumor Tissue Cohort includes men diagnosed with prostate cancer in these cohorts for whom archival formalin-fixed, paraffin embedded (FFPE) tumor specimens from prostatectomy and transurethral resection of the prostate have been retrieved (N = 2,200). For a subset of this cohort, we undertook a gene expression profiling study, sampling 404 men using an extreme case design, which includes all of the 113 men who died of prostate cancer or developed distant metastases and who had sufficient usable tumor tissue (lethal cases) and a sample of 291 men who lived at least eight years after cancer diagnosis and who neither developed metastases nor died of prostate cancer through 2012 (non-lethal cases). For the non-lethal cases we oversampled men for whom blood samples were available; their clinical characteristics were similar to those of the entire group of men with non-lethal disease.

RNA Extraction and Profiling

To conduct the profiling in FFPE tissue, whole transcriptome amplification was paired with microarray technologies. Briefly, RNA samples were extracted on a Biomek FxP automated platform using the Agencourt FormaPure FFPE kit [Cat #A33342] (Beckman Coulter Inc., Brea, CA). The mRNA was amplified using the WT-Ovation FFPE System V2 (Nugen, San Carlos, CA), a whole transcriptome amplification system that allows for complete gene expression analysis from FFPE samples known to harbor small and degraded RNA. Using a combination of 5' and random primer, reverse transcription created a cDNA/mRNA hybrid. The mRNA was subsequently fragmented, creating binding sites for DNA polymerase. Isothermal strand-displacement, using a proprietary DNA/RNA chimeric SPIA primer, amplified the cDNA. The cDNA was then fragmented and labeled with a terminal deoxynucleotidyl transferase covalently linked to biotin to prepare for microarray hybridization. The labeled cDNA was then hybridized to a GeneChip Human Exon 1.0 ST microarray (Affymetrics, Santa Clara, CA). A pilot study was conducted to validate the reliability and reproducibility of gene expression quantification from FFPE tissues on the study platform (20).

Pre-Processing

To process the data, we regressed out technical variables including mRNA concentration, age of the block, batch (96-well plate), percent of probes on the array detectable above the background, and log-transformed average background signal for each probe intensity of the raw data. The residuals were shifted to have the original mean expression values and normalized using the Robust Multi-array Average method (21). We mapped gene names to Affymetrix transcript cluster IDs using the NetAffx annotations as implemented in the Bioconductor annotation package *pd.hugene.1.0.st.v1*; this resulted in 20,254 unique named genes. Gene expression data are available through Gene Expression Omnibus accession number GSE62872.

Pathway Selection and Construction

We focused on expression in five molecular pathways with a suspected or confirmed link to stress: the adrenergic, muscarinic, glucocorticoid, dopaminergic, and serotoninergic signaling pathways. The majority of candidate genes in the pathways were selected using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Pathway Maps (Thomson Reuters). In addition, we searched the literature to identify any other genes related to both the selected pathways and cancer. Focusing on tumor-specific impact, genes in signaling branches leading exclusively to cardiovascular and neuronal functions were excluded from the adrenergic signaling pathway. In order to conservatively measure the pathway effect without crosstalk, 51 exclusive genes were defined for the five pathways (Supplementary Table S1) prior to all data analyses.

Clinical Data

Information on prostate-specific antigen (PSA) levels at diagnosis and tumor stage was abstracted from medical records and pathology reports, respectively. Hematoxylin and eosin slides from all cases underwent standardized histopathologic review including for Gleason

grade. For the majority of the cases in the HPFS, a single pathologist (M. Fiorentino) reviewed slides for the presence of perineural invasion, in which tumor envelops a peripheral nerve in cross section revealing tumor cell spread.

Immunohistochemistry

As secondary outcomes, we characterized tumor cell proliferation, apoptosis and angiogenesis using immunohistochemical methods.

Ki-67—As a classic marker of cell proliferation, the expression of Ki-67 was assessed on 5-micron sections of tumor tissue using a rabbit polyclonal antibody (Vector Labs, Burlingame, CA; diluted 1:1,500), as previously described (22). After immunohistochemical staining, the Ariol instrument SL-50 (Applied Imaging, San Jose, CA) was used to quantify the percentage of Ki-67-positive nuclei among all tumor nuclei.

Apoptosis—Using the Apoptag Peroxidase In situ kit (Chemicon International, Temecula, CA), the TUNEL assay was used on 5-micron sections to identify the proportion of tumor cells undergoing apoptosis, as previously described (22). Apoptosis was quantified as the percentage of positively-stained area over the whole tumor area by using Ariol instrument SL-50.

Angiogenesis—Protein expression of endothelial cell marker CD34 was ascertained on 5-micron sections in the HPFS 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), as previously described (23). Semi-automated image analysis, Image ProPlus 4.5 software (Media Cybernetics, MD), was used to quantify the size and architecture. Microvessel density was measured as the number of vascular structures in a high-powered field. Vessel size was determined as the average vessel diameter (μm), and area comprised by a vessel (μm^2).

Statistical Analysis

For each of the five candidate pathways, we assessed the overall association between the genes in the pathway and the risk of lethal prostate cancer using logistic regression. Because of the small pathway sizes (5 - 17 genes), we used a likelihood ratio test that compared the null model (adjusted for age at diagnosis and cohort) to the full model (including also the genes in the candidate pathway). Because our aim was to describe the differences observed in these pathways between lethal and non-lethal disease, rather than to build prognostic models, our primary model does not control for Gleason and stage, but we consider secondary models that control for and subset by these variables. We compared the results to the global test, which can be more sensitive than the likelihood ratio test to alternatives where many genes have small contributory effects (24). To explore possible interaction between genes within the pathways, we also tested for nonlinear and interaction effects using the global test with interaction terms and kernel smoothing terms (25). For pathways demonstrating significant associations, we further examined the impact of specific genes involved in those pathways in the logistic regression models.

We also performed a competitive pathway test, Gene Set Enrichment Analysis (GSEA) (26), in which the differential expression of the genes in the pathway is compared to differential expression of genes not involved in the pathway, using all 20,254 available named genes. This competitive approach offers an assessment of the importance of the pathways relative to associations between other unrelated genes and the outcome, but in order to calculate p-values based on permuting individuals in the study, this approach does not control for clinical covariates. It also provides a direction for the association, which illustrates whether up-regulation of the gene expression tends to be associated with lethality or non-lethality.

To determine whether differential expression of these pathways occurs globally in prostate tissue or is specific to tumors, we tested for associations in tumor tissue and adjacent normal prostate tissue separately. To test how tumor characteristics may modify the associations with lethality, we stratified the analyses by Gleason score (5-6, 7 and 8-10) and tumor stage (T1/T2 and T3/T4/N1/M1), and we also tested models controlling for Gleason and stage.

To further understand potential underlying mechanisms for the observed associations, we tested for pathway relationships with cell proliferation, apoptosis, and angiogenesis. Since the values of some biomarkers had skewed distributions, we categorized all biomarkers into quartiles; we then fit proportional odds models with the markers as outcomes, and assessed pathway significance using likelihood ratio tests. We also tested for associations with Gleason score and tumor stage using proportional odds models, creating ordinal variables out of Gleason score (5-6, 3+4, 4+3, and 8-10), stage (T1/T2, T3, and T4/N1/M1), and PSA levels at diagnosis (0-3.9 ng/ml, 4-9.9 ng/ml, and 10+ ng/ml). Finally, we considered whether the pathways were differentially expressed between cases that exhibited perineural invasion and those that did not.

Because we are considering five independent pathways, we considered a p-value of less than 0.01 to indicate statistical significance, to help correct for multiple testing. All analyses were performed in R (Version 3.0.2). This study was approved by the institutional review boards at the Harvard School of Public Health and Partners Health Care.

Results

After processing and removing cases that failed quality control, gene expression data was available on tumor tissue from 404 men, with matching normal tissue from 202 of these men. Clinical and biological characteristics of these men are provided in Table 1. A larger number of lethal cases were available from the HPFS, yielding slightly different distributions of Gleason score among patients selected from the two cohorts.

The pathway tests for lethal prostate cancer are shown in Table 2. We observed significant associations between lethal prostate cancer and differential signaling in prostate tumor tissue for the adrenergic ($p=0.001$), glucocorticoid ($p<0.0001$), serotoninergic ($p=0.0019$), and muscarinic ($p=0.0045$) pathways.

The glucocorticoid and serotoninergic pathways remained statistically significant in models controlling for Gleason and stage ($p=0.0006$ and $p=0.0062$, respectively), suggesting these pathway associations might be independent of tumor grade and stage. Differential signaling

was observed in both high and low stage tumors for the glucocorticoid pathway (p=0.0004 and p<0.0001, respectively), and was noted in prostate cancer cases with Gleason 8-10 (p=0.0002). Results were similar using the global test (data not shown). Tests for any gene-gene interactions and any nonlinear gene effects using global test methodology were not statistically significant at the 0.01 level, suggesting that interactions and nonlinearity are not critical factors in these pathway relationships (data not shown). None of the pathways was differentially expressed in the adjacent normal tissue of lethal prostate cancer compared with non-lethal prostate cancer. This was not simply due to reduced sample size because the associations in tumor tissue persisted even when we subset to men with matching normal tissue (results not shown).

When using the competitive pathway test (GSEA), only the adrenergic pathway (p=0.001) was statistically significant; up-regulation of genes in this pathway tended to be associated with non-lethal disease. Although this test does not control for clinical covariates, it provides a helpful somewhat complementary analysis to the primary self-contained pathway tests, because many pathways and processes are altered in prostate cancer pathogenesis and progression.

Table 3 shows the associations between pathway expression and biomarkers of cell proliferation, apoptosis, and angiogenesis, as well as pathological characteristics (Gleason score, tumor stage, and perineural invasion). We found that differential signaling in the glucocorticoid pathway was associated with ki-67 (p<0.0001), diameter of blood vessels (p=0.0001), and vessel area (p=0.0001). Differential signaling in the adrenergic pathway was observed between ki-67 quartiles (p=0.0061), and was suggested in vessel area (p=0.031) and size (p=0.025). The adrenergic, glucocorticoid, and muscarinic pathways were all differentially expressed across categories of Gleason score (p<0.0001 for adrenergic and glucocorticoid; p=0.0032 for muscarinic) but only the glucocorticoid pathway was differentially expressed across categories of stage (p=0.0034). Perineural invasion was associated with aberrant signaling in the glucocorticoid (p=0.0002) and serotoninergic pathways (p=0.0097).

Table 4 presents gene-level effects for the four pathways that were statistically significant overall for lethal prostate cancer. The adrenergic pathway had several genes with contributing effects, with ADRA1A showing the strongest single association (OR=0.21, p=0.0004). The glucocorticoid pathway was dominated by two genes with opposite associations: PTGES3 (OR=6.5, p<0.0001) and SMAD4 (OR=0.17, p<0.0001). The serotoninergic pathway appeared to contain many genes with moderate effects, though the HTR2B gene showed the strongest association with lethal disease (OR=4.0, p=0.0047). The muscarinic pathway was driven by CHRM1, which showed a negative association with lethal prostate cancer (OR=0.29, p=0.0001).

Discussion

Although there is evidence from experimental studies that neuroendocrine signaling may promote cancer progression, this is, to the best of our knowledge, the first large study on men with prostate cancer to comprehensively investigate the association between stress-

related signaling pathways and prostate cancer progression at the transcription level. We found differential gene expression in four out of five selected stress-signaling pathways in the tumors of men with lethal and non-lethal prostate cancer. Two pathways in particular emerge as being most interesting and worth further investigation – the adrenergic and glucocorticoid pathways – and we discuss these in further detail.

The adrenergic pathway is statistically significantly associated with lethality using both the self-contained pathway test (p=0.001) and the competitive pathway test (p=0.001). These two results suggest both that the genes in this pathway are significantly associated with lethal outcome in a standard logistic regression model and that their patterns of differential expression are unusual even when compared to the background of processes and pathways altered in prostate cancer development. The adrenergic pathway is no longer statistically significantly at our 0.01 cutoff when controlling for Gleason and stage, instead we see a strong association with categories of Gleason score (p<0.0001) and ki-67 (p=0.0061), suggesting that understanding the relationship between this pathway and cell dedifferentiation and proliferation in tumor pathogenesis could be a fruitful direction for further study.

Our finding that adrenergic pathway dysregulation is associated with lethal prostate cancer is in line with earlier studies of adrenaline and ADRB2 signaling as mediators of prostate cancer development and local invasion (5,7). Although we aimed to assess signaling in entire pathways rather than in individual genes, we examined ADRB2 individually, given previous interest in this receptor. We noted that, if anything, there seemed to be a negative association between ADRB2 expression and lethal prostate cancer at the transcription level. In contrast, expression of the ADRB2 receptor has been suggested to be higher in metastatic than in localized prostate cancer at the *protein* level (27). However, another study agrees with us that ADRB2 expression on the *transcription* level might be lower in metastatic than in localized prostate cancer (28). The discrepancy between *transcription* and *protein* levels of adrenergic signaling may simply imply a possible negative feedback loop due to long exposure to an agonist, such as chronic stress(29). Alternatively, the down-regulated mRNA level may be involved in de-differentiation and epithelial-mesenchymal transition with more potential to invade and migrate, as recently proposed by *Braadland et al* (17). A main contributor to the altered adrenergic pathway signaling in these data was the adrenergic receptor alpha-1D (ADRA1D), which has been implicated in prostate cancer cell proliferation earlier (30).

The glucocorticoid pathway demonstrated consistently strong associations with lethal prostate cancer even when controlling for Gleason and stage (p=0.0006). The pathway was also associated with cell proliferation (p=0.0001), angiogenesis (p=0.0001), perineural invasion (p=0.002), and additionally with Gleason and stage (p<0.0001 and p=0.0034, respectively). Although the glucocorticoid pathway was not statistically significant using GSEA, this may be in part because the most significant genes (PTGES3 and SMAD4) in the pathway had opposite associations with lethal prostate cancer, and GSEA is more powerful at detecting pathways with effects in the same direction.

A possible explanation for the relevance of glucocorticoid signaling to prostate cancer progression is the potential for a switch from androgen- to glucocorticoid-dependence observed in prostate cancer cells. In general, androgen supports tumor cell growth throughout the development and progression of prostate cancer. However, by activating mutated androgen receptors, *Zhao et al.* found that glucocorticoids could promote prostate tumor growth instead of androgen, producing androgen-independent growth (31). Glucocorticoids may also stimulate tumor growth and metastatic progression by suppressing the TGFβ/SMAD4 signaling axis (32).

Interestingly, none of the selected pathways was clearly associated with lethality in adjacent normal tissue obtained from the same cases. A possible explanation for this is the different responses to stress between tumor and normal cells. *Magnon et al*. reported that both sympathetic and parasympathetic nerve fibers, which locally release adrenaline and acetylcholine respectively, were denser in prostate cancer tissue than in adjacent normal tissue (5). This could partially explain differences in the adrenergic and muscarinic signaling pathways observed in tumor and normal tissue.

Our findings of relationships between the stress-related signaling pathways and cell proliferation, angiogenesis, and perineural invasion, may suggest possible mechanisms for associations between these pathways and lethal prostate cancer. *Thaker et al*. found that activation of the adrenergic signaling pathway promoted tumor proliferation and angiogenesis in an ovarian cancer model (33). *Magnon et al*. later confirmed the impact of adrenergic signaling on cell proliferation in a prostate cancer animal model (5). Although glucocorticoids are known to suppress tumor growth and angiogenesis in prostate cancer (12), glucocorticoid pathway signaling, which seemed to be the main survival pathway for androgen-independent prostate cancer, was associated with cell proliferation (34). The association with perineural invasion requires further investigation, though it may partially reflect prostate cancer lethality (35,36). A major strength of this study is the utilization of a fairly large group of men with gene expression, immunohistochemical markers, and extensive clinical annotation available. In particular, because most men do not die of prostate cancer even after recurrence (37), prostate cancer-specific death, the primary outcome of our study, has been recognized as a more reliable outcome for prostate cancer prognosis (38). Another major strength is that we used a pathway-focused approach to study whether stress-related signaling pathways were associated with prostate cancer progression. We investigate the pathway as a whole, rather than focusing on individual genes.

There are potential limitations of our study. One concern is the interactions of shared downstream genes between pathways. To address this, we selected a smaller number of genes specific to the pathway to ensure that genes would not overlap but rather reflect the signaling pathway of interest. Also, balancing the potential benefit of a wider gene selection against the consequential risk of overestimating statistical significance (and detecting false positive associations) we chose the more conservative approach. A second concern is that there are other stress-related signaling pathways than the five available for study here. Future studies could for example also include pathways mediating stress-immunity (e.g., the NF-κB signaling pathway) and other stress-psychiatric pathways (e.g., the brain-derived neurotrophic factor pathway). Moreover, although our interest in these pathways stemmed

from an interest in the role of psychological stress and cancer progression, we note that while it is possible that altered signaling could be the result of psychological stress (1,7), it could also be explained by post-translational modification. The mRNA differences captured may partially derive from the tumor microenvironment (such as peripheral neurons or stromal cells) or reflect manifestations of local paracrine control rather than the influence of psychological stress. However, whether the dysregulation is driven by systemic or tumor intrinsic factors, such signaling pathways may represent mechanisms targetable for intervention.

Finally, our approach uses gene expression assessed at a single point in time for each man, and because of this we are only provided with a biological snapshot to investigate differences in these pathways according to key clinical characteristics. This approach allows us to observe differences in these pathways, but does not allow us to make temporal or functional claims. The contribution of this paper, then, is to demonstrate that there are differences in some neuroendocrine pathways when comparing lethal and nonlethal cancers, as well as other clinical features, and to provide the guidance that the adrenergic and glucocorticoid pathways look most interesting to pursue. Future research is needed to confirm these associations, understand when alteration of these pathways occurs, and determine whether intervention through these signaling pathways could be effective for intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Growing experimental evidence suggests that neuroendocrine signaling may influence cancer progression. Whether driven by systemic or tumor intrinsic stress, such signaling pathways may represent mechanisms targetable for intervention. Leveraging a large cohort of US men with prostate cancer, we assessed mRNA expression of 51 genes involved in five predetermined stress-related signaling pathways in tumor tissue. We observed that genes in two key pathways – the adrenergic and glucocorticoid signaling pathways – exhibit dysregulation in the tumors of patients whose prostate cancer proves to be lethal; and in association with increased cell proliferation, angiogenesis, and perineural invasion. The current study lends support to the hypothesis that altered neuroendocrine signaling is associated with prostate cancer progression, and motivates further studies to understand the temporal and functional roles of these pathways in prostate cancer progression. Better understanding of these pathways may open up avenues for the development of new therapeutic intervention strategies for prostate cancer.

Table 1

Characteristics of Prostate Cancer Patients in the Physicians' Health Study and Health Professionals Follow-up Study cohorts - mean (SD) or median [Q1, Q3][*]

	PHS	HPFS
Total number	150	254
With normal tissue, N(%)	82 (55)	120 (47)
Years of diagnosis	1982-2005	1986-2004
Age at diagnosis, years (SD)	66 (6.5)	65 (6.4)
Lethal cases, N(%)	30 (20)	83 (33)
Gleason score, N(%)		
2-6	33 (22)	24 (9)
7 (3+4)	48 (32)	91 (36)
7 (4+3)	28 (19)	74 (29)
8-10	41 (27)	65 (26)
Tumor stage, N(%)[†]		
T1/T2, N0/Nx	89 (59)	150 (59)
T3, N0/Nx	49 (33)	83 (33)
T4, N1, M1	12 (8)	21 (8)
PSA at Diagnosis, N (%)		
0-3.9 ng/ml	15 (12)	18 (8)
4-9.9 ng/ml	79 (61)	119 (56)
10+ ng/ml	35 (27)	75 (35)
Cell proliferation- Ki-67 staining		
Percentage of positive nuclei	0.13 [0, 0.46]	0.23 [0.03, 1.09]
Apoptosis- TUNEL assay		
Percentage of stained area > 0.5	45 (45)	65 (42)
Angiogenesis- CD34 staining		
Microvessel density[‡]	-	68.8 [56.6, 96.8]
Vessel area, μm^2	-	423.0 [323.1, 549.9]
Diameter of blood vessels, μm	-	23.4 [20.8, 26.4]

	PHS	HPFS
Perineural Invasion	-	73 (55)

*21PHS and 42 HPFS were missing PSA levels at diagnosis; 29 PHS and 61 HPFS were missing Ki-67; 50 PHS and 99 HPFS were missing apoptosis; 80 HPFS were missing angiogenesis markers; 122 HPFS were missing perineural invasion assessment. N, number; SD, standard deviation; Q1, first quartile; Q3 third quartile; PSA, prostate-specific antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

†In the PHS, 18 men were missing pathological stage but had information on clinical stage, so we used this information as an approximation for pathological stage: 12 were cT1 or cT2, 1 was cT3, and 5 were cT4 or cN1 or cM1. In this HPFS, 17 men were missing this information, and we used their clinical stage: 9 were cT1 or cT2, 1 was cT3, 7 were cT4 or cN1 or cM1.

‡Microvessel density is the number of vessels per high-powered field.

Table 2

Pathway Tests for Lethal Outcome (*P* values) - Likelihood ratio tests comparing null and alternative models in logistic regression with lethal outcome. All null models include age and indicator of cohort membership (PHS vs. HPFS). Null models include Gleason and stage when indicated that these are controlled for. For the GSEA test, no control covariates are used.

Lethality	N	Adrenergic (11 genes)	Glucocorticoid (12 genes)	Dopaminergic (6 genes)	Serotoninergic (17 genes)	Muscarinic (5 genes)
Tumor Tissue	404	0.0010	<0.0001	0.053	0.0019	0.0045
Normal Tissue	202	0.31	0.29	0.58	0.37	0.70
Controlling for Gleason	404	0.058	0.0012	0.39	0.013	0.093
Controlling for Gleason and Stage	404	0.043	0.0006	0.31	0.0062	0.25
Among Gleason 5-6	57			no deaths		
Among Gleason 7	241	0.063	0.098	0.51	0.017	0.30
Among Gleason 8, 9, or 10	106	0.39	0.0002	0.16	0.062	0.078
Among Low Stage*	239	0.071	<0.0001	0.12	0.13	0.28
Among High Stage†	165	0.034	0.0004	0.31	0.088	0.084
GSEA test‡	404	0.001 (NL)	0.31 (L)	0.47 (NL)	0.58 (L)	0.22 (NL)

*Low stage defined as T1/T2, N0/Nx.

†High stage defined as T3/T4, N1, M1.

‡GSEA provides an indication of whether the pathway is up-regulated in either the lethal (L) or non-lethal (NL) phenotype – so the adrenergic pathway being up-regulated in non-lethal suggests that the preponderance of genes in that pathway are positively associated with non-lethality (i.e., higher expression of the genes is associated with a protective effect).

Table 3

Overall Pathway Tests for Secondary Outcomes (*P* values) - Likelihood ratio tests comparing null and alternative models in proportional odds model (for categorical outcomes) or logistic regression (for dichotomous outcomes). Null models include age and indicator of cohort membership (PHS vs. HPFS).

Secondary Outcomes	N	Adrenergic (11 genes)	Glucocorticoid (12 genes)	Dopaminergic (6 genes)	Serotoninergic (17 genes)	Muscarinic (5 genes)
Ki-67 (quartiles)	314	0.0061	<0.0001	0.096	0.49	0.46
Apoptosis (quartiles)	255	0.24	0.29	0.51	0.50	0.38
Microvessel density (quartiles)	174	0.13	0.21	0.19	0.31	0.88
Vessel area (quartiles)	174	0.031	0.0001	0.051	0.58	0.16
Diameter of blood vessels quartiles)	174	0.025	0.0001	0.10	0.79	0.15
Perineural Invasion (yes/no)	132	0.025	0.0002	0.032	0.0097	0.029
Gleason (4 categories)*	404	<0.0001	<0.0001	0.078	0.54	0.0032
Tumor Stage (3 categories)†	404	0.070	0.0034	0.52	0.66	0.14
PSA at Diagnosis (3 categories)‡	341	0.25	0.06	0.37	0.42	0.0024

*Gleason scores were categorized as 5-6, 3+4, 4+3, and 8-10.

†Tumor stages were categorized as T1/T2, N0/Nx; T3, N0/Nx; and T4, N1, Ml.

‡PSA (prostate-specific antigen) levels at diagnosis were categorized as 0-3.9 ng/ml, 4-9.9 ng/ml, and 10+ ng/ml.

Table 4

Gene-Level Relationships with Lethal Outcome, in logistic regression models including all genes in the pathway, as well as age at diagnosis and cohort membership as control covariates*

Adrenergic	ORs	*P*	Glucocorticoid	ORs	*P*	Serotoninergic	ORs	*P*	Muscarinic	ORs	*P*
ADRA1A	0.21	0.0004	FKBP4	1.2	0.63	HTR1A	0.5	0.19	CHRM1	0.29	0.0001
ADRA1B	0.98	0.97	HSP90AA1	2.0	0.090	HTR1B	0.28	0.074	CHRM2	1.3	0.70
ADRA1D	0.33	0.031	HSP90AB1	2.1	0.057	HTR1D	2.3	0.14	CHRM3	0.93	0.68
ADRA2A	1.1	0.87	HSPA1A	0.40	0.11	HTR1E	0.99	0.99	CHRM4	1.0	0.94
ADRA2B	0.98	0.97	HSPA1B	1.6	0.42	HTR1F	0.70	0.55	CHRM5	1.0	0.95
ADRA2C	0.41	0.15	HSPA4	0.51	0.12	HTR2A	4.1	0.027			
ADRB1	0.61	0.30	NR3C1	1.5	0.37	HTR2B	4.0	0.0047			
ADRB2	0.78	0.52	POU2F1	4.9	0.015	HTR2C	0.44	0.23			
ADRB3	0.75	0.55	POU2F2	0.74	0.69	HTR3A	1.4	0.55			
ADRBK1	0.48	0.046	PTGES3	6.5	<0.0001	HTR3B	2.8	0.088			
ADRBK2	0.76	0.33	SMAD3	0.75	0.56	HTR3C	0.97	0.96			
			SMAD4	0.17	<0.0001	HTR3D	0.49	0.24			
						HTR3E	2.8	0.085			
						HTR4	0.79	0.75			
						HTR5A	0.53	0.24			
						HTR6	0.31	0.024			
						HTR7	0.65	0.47			

*Odds ratio (OR) > 1 indicates over-expression of the gene, while OR<1 indicates under-expression.