Germline Variants in Asporin Vary by Race, Modulate the Tumor Microenvironment, and Are Differentially Associated with Metastatic Prostate Cancer

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Abstract

Purpose: Prostate cancers incite tremendous morbidity upon metastatic growth. We previously identified Asporin (ASPN) as a potential mediator of metastatic progression found within the tumor microenvironment. ASPN contains a aspartic acid (D)-repeat domain and germline polymorphisms in D-repeat-length have been associated with degenerative diseases. Associations of germline ASPN D polymorphisms with risk of prostate cancer progression to metastatic disease have not been assessed.

Experimental Design: Germline ASPN D-repeat-length was retrospectively analyzed in 1,600 men who underwent radical prostatectomy for clinically localized prostate cancer and in 548 noncancer controls. Multivariable Cox proportional hazards models were used to test the associations of ASPN variations with risk of subsequent oncologic outcomes, including metastasis. Orthotopic xenografts were used to establish allele- and stromaspecific roles for ASPN D variants in metastatic prostate cancer.

Results: Variation at the ASPN D locus was differentially associated with poorer oncologic outcomes. ASPN D14 [HR, 1.72; 95% confidence interval (CI), 1.05–2.81, P = 0.032] and heterozygosity for ASPN D13/14 (HR, 1.86; 95% CI, 1.03–3.35, P = 0.040) were significantly associated with metastatic recurrence, while homozygosity for the ASPN D13 variant was significantly associated with a reduced risk of metastatic recurrence (HR, 0.44; 95% CI, 0.21–0.94, P = 0.035) in multivariable analyses. Orthotopic xenografts established biologic roles for ASPN D14 and ASPN D13 variants in metastatic prostate cancer progression that were consistent with patient-based data.

Conclusions: We observed associations between ASPN D variants and oncologic outcomes, including metastasis. Our data suggest that ASPN expressed in the tumor microenvironment is a heritable modulator of metastatic progression.

Introduction

The burden of prostate cancer-related mortality remains significant due to the subset of prostate cancers that progress to metastatic disease (1). Identification and differentiation of patients with aggressive cancer from those with indolent disease remains a key issue in the management of clinically localized prostate cancer. To date, most biomarker studies have focused on carcinoma-specific features predictive of aggressive disease. As the tumor microenvironment influences prostate cancer progression (2), we hypothesized that molecules in the primary tumor microenvironment secreted by cancer-associated fibroblasts could modulate tumor progression and predict metastatic potential. We previously reported that Asporin...
Translational Relevance

In this study, we report that germline ASPN D-repeat-length variants expressed in the tumor microenvironment are differentially associated with progression to metastatic prostate cancer. To the best of our knowledge, this is the first report of an association of a germline variant expressed by nontumor cells in the tumor microenvironment with metastatic prostate cancer progression. We additionally show that ASPN expression is associated with local prostate cancer progression as measured by Gleason grade and poorer oncologic outcomes, including metastasis. We begin to establish biologic roles for ASPN D-repeat-length variants in progression to metastatic prostate cancer. This study highlights the potential clinical utility of using germline ASPN D-repeat-length to improve early therapeutic decisions for men who have localized cancer with lethal potential, as well as providing novel heritable and mechanistic insights into progression to lethal disease.

Materials and Methods

JHH germline study population

We retrospectively analyzed a database of 19,142 men who had undergone radical prostatectomy and pelvic lymphadenectomy at Johns Hopkins Hospital (JHH, Baltimore, MD) since 1992 (PSA era). Germline DNA was available for approximately 10,000 patients. We selected 1,672 cases for genomic analysis that were weighted for approximately equal Gleason grades and to generate approximately equal white and nonwhite race groups, of which 1,600 had adequate DNA for germline genotyping. 55.6% men self-reported Caucasian, 43.6% self-reported African American, and 0.8% self-reported other ethnicity or were of unknown ethnicity. We retrospectively analyzed 192 self-reported Caucasian and 370 self-reported African American male controls with no cancer, of which 179 and 369, respectively, had adequate germline DNA for genotyping. All participants provided written informed consent. The protocol and consent documents were approved by the Johns Hopkins University (JH, Baltimore, MD) Institutional Review Board (IRB).

Radical prostatectomy specimens were processed as previously described (13). Each tumor was graded using the Gleason scoring system and staged using the TNM (tumor–node–metastasis) system. Clinical outcome data included biochemical recurrence [BCR; defined as a postoperative prostate-specific antigen (PSA) ≥0.2 ng/mL], distant metastasis (defined as postoperative clinical or radiographic spread of disease to extrapelvic lymph nodes, bones, or viscera), and prostate cancer–specific mortality (PCSM). Outcome or time to outcome data were not available for all men in the cohort; any resulting differences in sample size number were recorded in the Results section.

Genomic DNA isolation

Tissue for genomic DNA isolation was taken from seminal vesicles at the time of radical prostatectomy (cases) or from blood of men without prostate cancer diagnoses (controls). Seminal vesicle tissue was suspended in 12 mL suspension buffer (20 mmol/L Tris; 25 mmol/L EDTA; 100 mmol/L NaCl) + 1 mL 10% SDS + 60 μL Proteinase K solution (20 mg/mL), inverted twice, and then incubated overnight at 50°C. The next day, DNA was digested by adding 60 μL RNase A Solution (Qiagen) and incubating at 37°C for 1 hour. Proteins were precipitated in 4 mL protein precipitation solution (Promega) on ice for 20 minutes and then centrifuged at 2,000 g for 10 minutes. DNA was extracted from the supernatant with 30 mL 100% isopropanol, centrifuged 2,000 × g for 5 minutes, and then washed with 70% ethanol followed by centrifugation.

Genotyping of the D-repeat polymorphism

The D-repeat polymorphism located in the N-terminal region of the ASPN gene was PCR amplified using 5′ primer 6-FAM-ATTCCTGCGGTTGCGTGCTC and 3′ primer TGGCTTCTTGCGTCCTCGT. Primers were designed using Oligo software. Reactions were carried out in 10 μL consisting of 30 ng DNA, 0.125 μmol/L primers, 0.6 mmol/L dNTPs (Contiental Lab Products), 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCL, 1.5 mmol/L MgCl, and 0.6 units of Taq Gold DNA polymerase (Perkin Elmer). Amplification was performed in a Veriti Thermal Cycler (Applied Biosystems Inc.) for an initial denaturation of 12 minutes at...
94°C followed by 40 cycles of 94°C for 20 seconds, 58°C for 20 seconds, 72°C for 30 seconds, and a final 10 minute elongation at 72°C. The PCR products were electrophoresed on an ABI 3730xl DNA Analyzer (Applied Biosystems Inc.). Data were collected and analyzed with GeneMapper software (Applied Biosystems Inc.) that calculates fragment length in reference to an internal lane standard (LIZ500). Three homozygous samples of different repeat-length were confirmed by Sanger sequencing. PCR products were sequenced using fluorescent dideoxy terminator method of cycle sequencing. Reactions were run on a 3730xl DNA Analyzer (Applied Biosystems Division) following Applied Biosystems protocols. Sequence data was analyzed using Sequencher Software (Gene Codes).

Statistical analysis
Characteristics of patient groups defined by distinct ASPN variations were compared. Exploratory data visualization was used to identify departure from normality. Means of continuous variables were compared by t tests. Nonnormally distributed variables were compared by Wilcoxon–Mann–Whitney rank-sum tests. Chi-square tests were used to examine the association of ASPN variants with biopsy and pathologic Gleason sum. In lieu of univariate models to test strengths of association, bivariate adjusting for race were performed due to clinical and genomic race based differences in the analyzed cohort. Bivariate and multivariable logistic regression analysis was performed to test strengths of association with ASPN variants and pathologic outcomes: extraprostatic extension (EPE), seminal vesicle invasion (SVI), and lymph node involvement (LNI). Similarly, bivariate and multivariable Cox proportional hazards models were used to test the associations of ASPN variations with risk of subsequent oncologic outcomes: BCR and metastasis. This study was under-powered to examine PCSM. For multivariable analyses, Gleason sum was categorized as ≤7 or ≥8 and clinical stage was categorized as nonpalpable (T1a, T1b, T1c) versus palpable (T2a, T2b, T2c, T3). Metastasis-free survival estimates were derived from Kaplan–Meier life tables with surgery at the zero starting point. Statistics were computed with Stata 11.0 (StataCorp).

Immunohistochemistry, immunofluorescence, and immunoblotting
For immunohistochemistry (IHC) and immunofluorescence (IF), formalin-fixed paraffin-embedded (FFPE) tissues were sectioned, deparaffinized, steamed in Target Retrieval Solution Ready to Use (Dako) for 40 minutes, blocked with Protein Block Serum-Free (Dako), incubated with antibodies against ASPN (Sigma Prestige HPA008425; 1:400), and pan-cytokeratin (Sigma C2562; 1:400) in Antibody Diluent (Invitrogen). Sigma Prestige HPA008425 ASPN antibody was validated for immunohistochemistry and immunoblotting in accordance with JHU pathology TMA criteria (Supplementary Fig. S1A–S1E). For IHC, primary antibodies were followed by secondary antibodies (Vector Laboratories) and then detected with 3,3'-Diaminobenzidine kit (Vector Laboratories). For IF, primary antibodies were followed by Alexa Fluor Dye secondary antibodies (Invitrogen) and mounted with Vectashield hard-set mounting medium with DAPI counterstain (Vector Laboratories). Images were captured at room temperature on a Nikon E800 fluorescence microscope with 40× Plan Apo objective and a Nikon DS-QiMc camera with Nikon Elements imaging software (var 3.0).

For immunoblotting, lysates were fractionated on NuPAGE gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes, blocked, and then incubated with primary antibodies. Antibodies used included: ASPN (Sigma Prestige HPA008425; 1:1,000) and GAPDH (Santa Cruz sc-32233; 1:10,000). Blots were developed using enhanced chemiluminescence (Thermo Fisher Scientific).

Quantitative real-time PCR
Total RNA was purified using RNeasy Mini Kit Plus (Qiagen). First-strand cDNA was synthesized using random hexamer primers (Applied Biosystems) and Ready-To-Go You-Prime First-Strand Beads (GE Healthcare), according to the manufacturer’s instructions. Quantitative PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) with TaqMan primers specific to human or to mouse ASPN (Applied Biosystems). Applied Biosystems software was used to calculate threshold cycle values for ASPN and the reference genes HPRT (Applied Biosystems) and GAPDH (Applied Biosystems).

JHU and Harvard University tissue microarrays
JHU Gleason grade tissue microarrays (TMA) were constructed from archival tissue from radical prostatectomy performed at JHU between 2000 and 2001. Cases for the TMAs were reviewed and selected by a genitourinary pathologist. The index lesion was defined as the largest tumor of the highest grade. In each case, both benign adjacent glands and the index tumors of Gleason sum 5, 6, 7, 8, and 9 were spotted in triplicate.

The Harvard Health Professional Follow-Up Study TMAs have been described previously (14). TMAs were constructed from archival FFPE prostate tissue from men treated with radical prostatectomy for prostate cancer who were participants in the HPFS, and included 715 evaluable prostate cancer cases, diagnosed between 1983 and 2004. The mean follow-up time from diagnosis was 13 years. Tumor tissue from radical prostatectomy was reviewed by multiple pathologists to provide uniform evaluation of Gleason score and to identify areas of high-density tumor for construction of TMAs. At least three tumor cores were sampled from each case from the index lesion, which was defined as the largest tumor of the highest grade.

Four-micron-thick sections were stained for ASPN by IHC. Cases were scored by urologic pathologists for ASPN expression. Using established scoring schemes, ASPN intensity was evaluated and assigned an incremental score of 0 (negative), 1 (weak), 2 (moderate), or 3 (strong; ref. 15). For the JHU Gleason grade TMAs, the extent of staining was assigned a score for 0% to 25% (score 0), 26% to 50% (score 1), 51% to 75% (score 2), and 76% to 100% (score 3). For each sample, an ASPN score was calculated by adding the intensity score and the extent score (H-score). For the Harvard HPFS TMAs, the extent of staining was assigned a percentage from 0% to 100%. For each sample, an ASPN score was calculated by multiplying the intensity score and the extent score (H-score). H-scores were compared using one-way ANOVA with pairwise comparisons. Statistical tests performed using GraphPad Software were two-sided and P values less than 0.05 were considered statistically significant.
Mayo Clinic progression analyses

Methods including tissue preparation, RNA extraction, microarray hybridization, and microarray expression analysis have been previously described (15, 16).

Study design. Patients were selected from a cohort of high-risk radical prostatectomy patients from the Mayo Clinic with a median follow-up of 8.1 years. The cohort was comprised of 1,010 high-risk men that underwent radical prostatectomy between 2000 and 2006, of which 73 patients developed metastatic disease as evidenced by positive bone or CT scan. High-risk was defined as preoperative PSA >20 ng/mL, pathologic Gleason score 8–10, SVI, or GPSM score ≥10. The subcohort incorporated all 73 metastatic patients and a 20% random sampling of the entire cohort.

Statistical analysis. The summarized expression of core transcript cluster, 3214845, was used to represent the expression of ASPN. PAM (Partition Around Medoids) unsupervised clustering method was used on the expression values of all clinical samples to define two groups of high and low expression of ASPN. Statistical analysis on the association of ASPN with clinical outcomes was done using two endpoints: BCR, defined as two consecutive PSA values ≥0.2 ng/mL after radical prostatectomy and metastasis (M&T), as defined by a positive bone scan and/or CT/MRI evidence of metastatic disease.

Stable cell lines

Quick Change Site Directed Mutagenesis Kit (Agilent Technologies) was used to generate ASPN D13 and ASPN D14 cDNAs from parent human ASPN D17 cDNA (Origene). ASPN D13 and ASPN D14 were subcloned into pIRESNeo3 (Clontech). Stable cell lines were generated through transfection with Fugene (Roche) of cDNAs into WPMY1 (ATCC low passage) according to the manufacturer's instruction, plating at limiting dilutions, and isolating single clones. WPMY1 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and PS (Invitrogen).

Orthotopic xenografts

All animal experiments were approved by the Institutional Animal Care and Use Committee at JHU. A total of 500,000 WPMY1, WPMY1 ASPN D13 (A), and WPMY1 ASPN D14 (A) cells were mixed with 500,000 PC3 cells (authentic at JHU in September 2014) in Matrigel (BD Biosciences) at a 1:1 ratio. Cells were grafted into the prostate of male NOD scid gamma (NSG) mice (JHU) that were 10 weeks in age. Seven mice were used for each group. An isoflurane gas anesthetic system (Caliper Life Sciences) was used for surgical anesthesia. Mice were induced by isoflurane gas anesthetic system (Caliper Life Sciences) and then monitored daily for distress. One mouse with a WPMY1/PC3 orthotopic xenograft from experiment B was excluded from final analyses due to cell leakage into the body cavity as evidenced by over 30 metastatic lesions on the body cavity wall.

Results

Germline ASPN D-repeat-length variants and adverse prostate cancer clinical outcomes

To determine whether ASPN D-repeat-length correlates with prostate cancer progression to metastasis, ASPN D-repeat-length was examined in 1,600 men who underwent radical prostatectomy for clinically localized prostate cancer at JHH (Table 1). Men in this study had ASPN D-repeat-lengths ranging from 10 to 19 aspartate residues with most (approximately 90%) of men having alleles containing 13, 14, 15, or 16 residues (Supplementary Fig. S2). The most common ASPN D-length genotypes in JHH men with prostate cancer were (where the integer corresponds to the number of aspartate-coding repeats in a single allele of ASPN): 13/15, 13/13, 15/15, 14/15, and 13/14 with the remaining genotypes between 0.1% and 5.8% of the study population (Supplementary Table S1). To allow for adequately-powered comparative tests, genotypes present in greater than 10% of the population were selected for comparison to all other genotypes in the study population. Furthermore, single ASPN D-repeat-length alleles (either homozygous or heterozygous) present in greater than 10% of the population were also analyzed: Any 13, Any 14, and Any 15. Similar to prior reports (7, 9, 17, 18), the distribution of ASPN D-repeat-length varied with ethnicity (Supplementary Fig. S2; Supplementary Table S1), and thus all analyses were either matched or controlled for race.

Germline ASPN D-repeat-length variants and adverse prostate cancer clinical outcomes

We examined the relationship between ASPN-D-repeat length and metastatic disease progression following local therapy for prostate cancer. Through Cox regression analyses, we found that germline ASPN D13/14 was significantly associated with metastatic recurrence following surgery (HR, 2.02; 95% CI, 1.16–3.53;
Germline ASPN Variants and Metastatic Progression

**Table 1.** Baseline characteristics and cancer outcomes of JHH prostate cancer cases

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Median age (years; IQR)</th>
<th>Caucasian</th>
<th>African-American</th>
<th>Other or unknown</th>
<th>Median PSA (ng/mL; IQR)</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>1600</td>
<td>58 (53–63)</td>
<td>889 (55.6%)</td>
<td>697 (43.6%)</td>
<td>14 (0.8%)</td>
<td>5.59 (4.2–8.3)</td>
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<td>Clinical stage</td>
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<tr>
<td>T1</td>
<td>1,118 (69.9%)</td>
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<td>T2</td>
<td>427 (26.7%)</td>
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<td>T3</td>
<td>13 (0.8%)</td>
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<td>Unknown</td>
<td>42 (2.6%)</td>
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<tr>
<td>Biopsy Gleason ≤6</td>
<td>814 (51.0%)</td>
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<td>7 (3 + 4)</td>
<td>305 (19.1%)</td>
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<td>7 (4 + 3)</td>
<td>215 (13.5%)</td>
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<td>8</td>
<td>171 (10.7%)</td>
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<td>9-10</td>
<td>91 (5.7%)</td>
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<tr>
<td>Pathologic stage</td>
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<tr>
<td>pT2N0</td>
<td>921 (57.8%)</td>
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<tr>
<td>pT3aN0</td>
<td>461 (28.9%)</td>
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<tr>
<td>pT3bN0</td>
<td>137 (8.6%)</td>
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<tr>
<td>pN1</td>
<td>75 (4.7%)</td>
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<tr>
<td>Pathologic Gleason ≤6</td>
<td>556 (34.9%)</td>
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<tr>
<td>7 (3 + 4)</td>
<td>366 (22.9%)</td>
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<tr>
<td>7 (4 + 3)</td>
<td>153 (9.6%)</td>
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<td>8</td>
<td>273 (17.1%)</td>
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<tr>
<td>9-10</td>
<td>248 (15.5%)</td>
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<tr>
<td>Positive surgical margin</td>
<td>294 (18.5%)</td>
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<tr>
<td>Median follow-up (years; IQR)</td>
<td>3.0 (2.0–6.0)</td>
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<tr>
<td>Biochemical recurrence (BCR)</td>
<td>269/1,115 (24.1%)</td>
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<tr>
<td>Median time to BCR (years; IQR)</td>
<td>3.0 (1.0–5.0)</td>
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<tr>
<td>Metastasis (Met)</td>
<td>83/1,097 (7.6%)</td>
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<tr>
<td>Metastasis time to Met (years; IQR)</td>
<td>3.0 (2.0–6.0)</td>
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<tr>
<td>PSCM</td>
<td>37/1,133 (3.3%)</td>
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<td>Median time to PSCM (years; IQR)</td>
<td>6.0 (3.0–10.0)</td>
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</table>

*P = 0.013* even when adjusted for preoperative variables and race (HR, 1.86; 95% CI, 1.03–3.35; *P = 0.040*; Tables 2 and 3). Consistent with this, Kaplan–Meier survival estimates demonstrated that heterozygous ASPN D13/14 was a significant germline prognostic marker for worse metastasis-free survival (Fig. 1). In addition, germline carriers of the ASPN D14 allele were significantly more likely to progress to metastatic disease by multivariable analyses compared with all other alleles (HR, 1.72; 95% CI, 1.05–2.81; *P = 0.032*; Tables 2 and 3). These data suggest that germline ASPN D13/14 or any ASPN D14 is a risk factor for metastatic recurrence following surgery.

**Table 2.** Predictors of metastatic recurrence: bivariate cox regression analyses adjusted for race of the most common germline ASPN D genotypes/alleles compared with all other genotypes/alleles

<table>
<thead>
<tr>
<th>Genotype/allele</th>
<th>Metastatic recurrence (n = 1,082)</th>
</tr>
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<tbody>
<tr>
<td>13/13</td>
<td>0.51 (0.24–1.07)</td>
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<tr>
<td>13/14</td>
<td>2.02 (1.36–3.53)</td>
</tr>
<tr>
<td>13/15</td>
<td>0.99 (0.55–1.78)</td>
</tr>
<tr>
<td>14/15</td>
<td>1.20 (0.54–2.65)</td>
</tr>
<tr>
<td>15/15</td>
<td>0.45 (0.14–1.46)</td>
</tr>
<tr>
<td>Any 13</td>
<td>1.28 (0.76–2.16)</td>
</tr>
<tr>
<td>Any 14</td>
<td>1.61 (1.00–2.58)</td>
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<tr>
<td>Any 15</td>
<td>0.82 (0.51–1.32)</td>
</tr>
</tbody>
</table>

**Table 3.** Predictors of metastatic recurrence: multivariable cox regression analyses of germline ASPN D13/14, ASPN D14, and ASPN D15/13 compared with all other genotypes/alleles

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Multivariables (n = 1,052)</th>
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<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>13/14</td>
<td>1.86 (1.03–3.35)</td>
</tr>
<tr>
<td>13/14</td>
<td>1.72 (1.05–2.81)</td>
</tr>
<tr>
<td>13/13</td>
<td>0.44 (0.21–0.94)</td>
</tr>
<tr>
<td>Biopsy Gleason Sum</td>
<td>3.47 (2.07–5.81)</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>4.02 (2.55–6.90)</td>
</tr>
<tr>
<td>PSA</td>
<td>1.05 (1.03–1.08)</td>
</tr>
<tr>
<td>Age</td>
<td>1.01 (0.97–1.05)</td>
</tr>
<tr>
<td>Gland volume</td>
<td>1.01 (1.00–1.02)</td>
</tr>
<tr>
<td>Year of surgery</td>
<td>1.03 (0.97–1.10)</td>
</tr>
<tr>
<td>Race</td>
<td>0.74 (0.38–1.44)</td>
</tr>
</tbody>
</table>

NOTE: Boldfaced values represent statistically significant HRs. Abbreviation: RAF, risk allele frequency.

*Multivariable adjusted for Biopsy Gleason Sum, clinical stage, PSA, age, gland volume, year of surgery, and race.

In contrast to germline carriers of ASPN D13/14 or any ASPN D14, multivariable Cox regression analyses demonstrated that homozygous ASPN D13 (ASPN D13/13) was significantly associated with a reduced risk of metastatic progression following surgery (HR, 0.44; 95% CI, 0.21–0.94; *P = 0.035*; Table 3). Kaplan–Meier survival estimates demonstrated that germline carriers of ASPN D13/13 had better metastasis-free survival than men with germline ASPN D13/14 (Fig. 1). When compared with germline ASPN D13/13, men with germline ASPN D13/14 had over a four-fold higher hazard of metastatic progression (HR, 4.53; 95% CI, 1.65–12.42; *P = 0.003*; Supplementary Table S2). While ASPN D13/13 remained significantly protective when controlled for race, χ² analysis demonstrated that significantly fewer African American men carried the ASPN D13/13 genotype (*P = 0.0001*; Supplementary Table S1).

Given the association of BCR with metastatic progression (19), we evaluated the relationship between ASPN D-repeat-length and BCR. When compared with all other genotypes, bivariate analyses controlled for race demonstrated that ASPN D13/14 was significantly prognostic of BCR following surgery (HR, 1.43; 95% CI, 1.01–2.03; *P = 0.042*; Supplementary Table S3); however, when controlled for presurgical variables in multivariable Cox regression analyses, the hazard ratio associated with ASPN D13/14 only approached significance (Supplementary Table S4).

Germline ASPN D-repeat-length variants and adverse prostate cancer pathology

Both pre- and posttreatment clinical findings are used by the NCCN to stratify risk of disease recurrence and to guide treatment recommendations (20). Logistic regression analyses demonstrated that none of the genotypes examined were significantly associated with biopsy or pathologic Gleason grade, PSA, local invasion beyond the prostate (EPE), or invasion into the seminal vesicles (SVI; Supplementary Table S5). However, logistic regression analyses demonstrated that ASPN D13/14 was significantly associated with prostate cancer involving the lymph nodes at surgery (OR, 2.52; 95% CI, 1.44–4.42; *P = 0.001*; Supplementary Table S6). Multivariable logistic regression analyses adjusted for preoperative variables and race demonstrated that men with ASPN D13/14 (OR, 2.42; 95% CI, 1.29–4.56; *P = 0.006* or
Interestingly, elevated levels of ASPN expression were significantly elevated in multiple other cancers, including breast, colon, and pancreatic, compared with normal tissue. While low Gleason grade tumors (sum ≤6) rarely progress to metastasis, high Gleason grade tumors (sum 8–10) can have poor oncologic outcomes, even after radical prostatectomy (21). Immunohistochemical analysis of ASPN protein expression in TMAcs from IJU (15) and Harvard (22) demonstrated that ASPN was associated with increasing Gleason grade with the highest expression seen in PCAFs surrounding Gleason sum 8–9 tumors (Fig. 2D and Supplementary Fig. S3D). Consistent with protein expression, analysis of ASPN mRNA expression in a cohort from Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY; ref. 23) demonstrated that ASPN was significantly elevated in samples from Gleason sum 8–9 tumors (Supplementary Fig. S3E).

**ASPN expression and prostate cancer outcomes**

To determine whether ASPN expression in the primary tumor microenvironment was associated with disease progression, we examined ASPN mRNA expression in prostate cancer cohorts from MSKCC (23) and the Mayo Clinic (15, 24) and ASPN protein expression in a cohort from Harvard (22). ASPN expression was significantly associated with BCR in all three cohorts as demonstrated by Kaplan–Meier survival curves and/or Cox regression analyses (Fig. 2E and Supplementary Fig. S4A and S4B). While Kaplan–Meier analysis was not available for the MSKCC cohort due to metastatic event time being unavailable, Kaplan–Meier survival curves demonstrated that ASPN expression was significantly associated with metastatic recurrence in the Mayo Clinic cohort (Fig. 2F). Univariate analyses also demonstrated a significant association between ASPN expression and metastatic recurrence in both the MSKCC and Mayo Clinic cohorts; however, this was not significant in the Harvard cohort (Supplementary Fig. S4C and S4D).

**ASPN D-repeat domain variants in animal models of metastatic progression**

Germline ASPN D-repeat domain variants in animal models of metastatic progression. While Kaplan–Meier analysis was not available for the MSKCC cohort due to metastatic event time being unavailable, Kaplan–Meier survival curves demonstrated that ASPN expression was significantly associated with metastatic recurrence in the Mayo Clinic cohort (Fig. 2F). Univariate analyses also demonstrated a significant association between ASPN expression and metastatic recurrence in both the MSKCC and Mayo Clinic cohorts; however, this was not significant in the Harvard cohort (Supplementary Fig. S4C and S4D).

Germline ASPN D-repeat variants in men with prostate cancer and noncancer diagnoses

Polymorphisms in ASPN D-repeat-length have reported associations with susceptibility to diseases such as osteoarthritis and lumbar disc degeneration (9, 10). To determine whether ASPN D-repeat length was associated with prostate cancer incidence, we compared ASPN D-repeat-length in prostate cancer cases and noncancer male controls matched for race. The distribution of ASPN D-repeat-length in JHU race-matched prostate cancer cases and controls was nearly identical with none of the alleles or genotypes being statistically different by chi-square analyses (Supplementary Fig. S2; Supplementary Table S1) suggesting that D-repeat-lengths are not associated with prostate cancer incidence.

**ASPN expression in cancer-associated fibroblasts**

Our data suggest that germline ASPN-repeat-length variants are differentially associated with metastatic progression; however, little is known about the biologic role of ASPN in cancer progression. To better understand this, we first analyzed human TMAcs and demonstrated that ASPN expression was not elevated in tumor cells but rather was significantly elevated in PCAFs and noncancer male controls matched for race. The distribution of ASPN D-repeat-length in JHH race-matched prostate cancer cases and controls was nearly identical with none of the alleles or genotypes being statistically different by chi-square analyses (Supplementary Fig. S2; Supplementary Table S1) suggesting that ASPN D allelic repeat-lengths are not associated with prostate cancer incidence.

Germline ASPN D-repeat-length variants in men with prostate cancer and noncancer diagnoses

Polymorphisms in ASPN D-repeat-length have reported associations with susceptibility to diseases such as osteoarthritis and lumbar disc degeneration (7, 9). To determine whether ASPN D-repeat length was associated with prostate cancer incidence, we compared ASPN D-repeat-length in prostate cancer cases and noncancer male controls matched for race. The distribution of ASPN D-repeat-length in JHH race-matched prostate cancer cases and controls was nearly identical with none of the alleles or genotypes being statistically different by chi-square analyses (Supplementary Fig. S2; Supplementary Table S1) suggesting that ASPN D allelic repeat-lengths are not associated with prostate cancer incidence.
interactions. We stably overexpressed *ASPN* D14 and *ASPN* D13 in an immortalized human prostate fibroblast cell line derived from stroma adjacent to benign glands (WPMY1; Fig. 3A). While WPMY1 are germline homozygous for *ASPN* D13, they did not express *ASPN* in vitro (Fig. 3A). Two independent stable clones of both WPMY1 overexpressing *ASPN* D13 [WPMY1 *ASPN* D13 (A) and (B)] and WPMY1 overexpressing *ASPN* D14 [WPMY1 *ASPN* D14 (A) and (B)] were generated. Parental WPMY1, WPMY1 *ASPN* D13, and WPMY1 *ASPN* D14 were mixed with PC3 cells and then grafted into murine prostates. Expression of *ASPN* polymorphisms did not affect average orthotopic xenograft size as measured by GI weight (Fig. 3B and C); however, *ASPN* polymorphisms significantly affected the number of metastases (Fig. 3D and E). Because of the differences in 5-year survival rates between regional and distant prostate cancer in humans (1), we quantified the number of metastases to distant lymph nodes and other organs, including lung, liver, and pancreas (Fig. 3D).

**Figure 2.**

*ASPN* expression in prostate cancer-associated fibroblasts is associated with Gleason grade and oncologic outcomes. A, *ASPN* expression as determined by IHC in prostate tissue following radical prostatectomy. B, *ASPN* expression in prostate cancer-associated fibroblasts in prostate tissue following radical prostatectomy as determined by IF of *ASPN* and pancytokeratin at 400× magnification. C, *ASPN* expression in human primary prostate cancer-associated fibroblasts (PCAF), human primary benign prostate associated fibroblasts (BAF), an immortalized human benign prostate fibroblast cell line (WPMY1), primary human benign prostate epithelial cells (PrEC), and human prostate cancer cell lines (LNCaP, 22RV1, PC3) as determined by qRT-PCR (top) and immunoblotting (bottom). D, *ASPN* expression as determined by IHC in JHU TMAs containing stroma adjacent to benign (*n* = 48), Gleason sum ≤6 (*n* = 31), Gleason sum 7 (*n* = 6), and Gleason sum 8-9 (*n* = 27) prostate tissue. Statistical analysis performed by one-way ANOVA with Tukey multiple comparison test (mean ± SEM; **P** < 0.001; ***P*** < 0.0001; top). *ASPN* staining in human prostate cancer sections at 200× magnification (bottom). E, *ASPN* expression is associated with biochemical recurrence (BCR). Kaplan–Meier survival curves of *ASPN* in MSKCC and in Mayo High Risk Cohort for BCR-free survival. F, *ASPN* expression is associated with metastatic recurrence. Kaplan–Meier survival curves of *ASPN* in Mayo High Risk Cohort for metastatic recurrence.
and E). Both gross inspection and pathologic analyses supported that metastatic obstruction of the bile duct led to jaundice in approximately 33% of the WPMY1 ASPN D14 mice, while, conversely, jaundice was not detected in WPMY1 or WPMY1 ASPN D13 mice. Histologic examination demonstrated micrometastatic disease in WPMY1 ASPN D13 animals compared with the much larger visual metastases in WPMY1 ASPN D14 animals (Fig. 3E). Collectively, these studies suggest that ASPN polymorphisms may have both prognostic and biologic roles in disease progression.

Discussion

Significant progress has been made in the past several years to identify genetic risk factors for prostate cancer. Genome-wide association studies (GWAS) have identified many single nucleotide polymorphisms associated with prostate cancer incidence (25, 26); yet, few inherited determinants of aggressive prostate cancer have been identified (27, 28). Furthermore, associations of germline variants expressed by nontumor cells in the tumor microenvironment with metastatic prostate
cancer have not been described. Based upon its association with other diseases, we report a hypothesis-driven study to determine whether germline variants in ASPN D-repeat-length were differentially associated with metastatic prostate cancer. The distribution of ASPN D-repeat-length variants was similar between prostate cancer cases and race matched controls, supporting the absence of an association between ASPN D variants and prostate cancer incidence. This is consistent with prior GWAS or family-based linkage studies that have not found a strong association between this genomic region and prostate cancer risk (29–31). However, two common variants in ASPN D-repeat-length affected the risk of having metastatic disease. Multivariable analyses demonstrated that homozygous ASPN D13/13 was significantly protective of metastatic recurrence following surgery. Conversely, germline genotype ASPN D13/14 and the ASPN D14 allele were significantly prognostic of lymph node involvement and metastatic recurrence following surgery. While previous studies have not reported an association of this genomic region with metastatic prostate cancer, this may be due to limitations of prior studies that examined candidate genes (27) or genes associated with incidence (32). Consistent with our patient-based findings, an animal model of prostate cancer metastasis supports that ASPN D14 specifically expressed in PCAs promotes metastatic prostate cancer progression while ASPN D13 hinders metastatic progression.

Development of a minimally invasive and cost-effective genomic test for the early identification of aggressive prostate cancer has the potential to impact both prognostic and therapeutic decision making in localized prostate cancer. Further understanding of the biologic role of ASPN in the stroma has the potential to impact both prognostic and therapeutic development.

Disclosure of Potential Conflicts of Interest

D.M. Berman is a consultant/advisory board member for Metamark Genetics. A.E. Ross is a consultant/advisory board member for GenomDX. No potential conflicts of interest were disclosed by the other authors.

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