Elevated Expression of Angiogenin in Prostate Cancer and Its Precursors

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Abstract

Purpose: Angiogenin is a polypeptide involved in the formation and establishment of new blood vessels necessary for growth and metastasis of numerous malignant neoplasms, including prostatic adenocarcinoma. Antiangiogenin therapy inhibits the establishment, growth, and metastasis of prostatic adenocarcinoma in animal studies. In this study, we have investigated the expression of angiogenin in prostatic adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and adjacent benign prostatic epithelium in a large cohort of prostatectomy specimens.

Methods: We have studied the expression of angiogenin by immunohistochemistry in prostatic adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and adjacent benign prostatic tissue in 107 human total prostatectomy specimens.

Results: The percentage of cells staining positively for angiogenin in benign prostatic glandular epithelium (mean = 17%) was significantly less than for high-grade prostatic intraepithelial neoplasia (mean = 58%, \( P < 0.001 \)) and prostatic adenocarcinoma (mean = 60%, \( P < 0.001 \)). Compared with adjacent benign prostatic epithelium, the staining intensity was significantly greater in high-grade prostatic intraepithelial neoplasia (\( P < 0.001 \)) and prostatic adenocarcinoma (\( P < 0.001 \)). Furthermore, staining intensity has significantly stronger in prostatic adenocarcinoma versus high-grade prostatic intraepithelial neoplasia (\( P = 0.0023 \)). However, there was no correlation of angiogenin expression with various clinical and pathologic variables examined, including age at surgery, Gleason scores, pathologic stage, tumor extent, angiolymphatic invasion, extraprostatic extension, seminal vesical invasion, lymph node metastasis, surgical margin status, presence of prostatic intraepithelial neoplasia, and perineural invasion.

Conclusion: Angiogenin expression in prostatic tissue increases as prostatic epithelial cells evolve from a benign to an invasive phenotype. The increasing expression of prostatic adenocarcinoma in the progression from benign prostate to high-grade prostatic intraepithelial neoplasia and ultimately to prostatic adenocarcinoma are consistent with previous studies showing the influential role that angiogenin plays in the growth, invasion, and metastasis of prostatic adenocarcinoma and many other malignant tumors.

Tumor angiogenesis, also termed neovascularization, provides a critical component for the growth, invasion, and metastasis of solid tumors, including prostatic adenocarcinoma (1–5). Angiogenin, a 123-amino-acid, 14.1-kDa polypeptide, is active in the regulation of angiogenesis in both physiologic and pathologic states (6–12). Multiple studies have shown increased expression of angiogenin, as measured by immunohistochemistry, in situ hybridization, or serum concentration, in numerous human malignancies, including colon, gastric, head and neck squamous, hepatocellular, pancreatic, and uterine carcinomas; melanoma; and gestational trophoblastic tumors (13–22). Moreover, in the many of these cancers, prognosis was correlated with the degree of angiogenin expression (13–17, 19). In prostatic adenocarcinoma, an increase in blood vessel density, a measurement of angiogenesis, has been correlated with a poor prognosis (23, 24).

Inhibition of the growth, progression, and metastasis of prostatic adenocarcinoma has been shown using several antiangiogenic modalities. These inhibitory techniques include blocking antibodies specific to angiogenin, antisense oligonucleotide blockade of angiogenin, and small molecule inhibitors of other angiogenic peptides (4, 5, 24, 25). Specifically, angiogenin antagonists have been shown to inhibit tumor growth (4, 5, 24, 25). Furthermore, in animal models using human prostate cancer cell lines in athymic mice, blocking antibodies and antisense oligonucleotide targeting against angiogenin have been shown to prevent establishment, progression, and metastasis of prostate cancer (4, 25). Recent
evidence indicates that angiogenin may be a "crossroad" in the angiogenic pathway involving acidic and basic fibroblast growth factor, vascular endothelial growth factor, and epidermal growth factor (27).

In this study, we have used a large cohort (n = 107) of radical prostatectomy specimens to investigate the degree of angiogenin expression by immunohistochemistry in prostatic adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and adjacent benign prostatic tissue. Additionally, the level of angiogenin expression was correlated with multiple clinical and pathologic variables, including age at surgery, Gleason scores, pathologic stage, tumor extent, angiolymphatic invasion, extraprostatic extension, seminal vesical invasion, lymph node metastasis, surgical margin status, presence of prostatic intraepithelial neoplasia, and perineural invasion.

Materials and Methods

Patients. Radical prostatectomy specimens (n = 107) containing invasive prostatic adenocarcinoma with adjacent high-grade prostatic intraepithelial neoplasia and normal prostatic glandular epithelium were obtained from the surgical pathology files of Indiana University Medical Center from 1990 to 1999. These cases were chosen to include the entire spectrum of Gleason grade and pathologic stages. The patients ranged in age from 44 to 77 years (mean = 63 years). Grading of the primary tumor from radical prostatectomy specimens was done according to the Gleason system (28). The Gleason scores ranged from 4 to 10. Pathologic staging was done according to the 1997 tumor, lymph nodes, and metastasis system (29). The final pathologic stages included T\textsubscript{1} (54 patients), T\textsubscript{2}a (30 patients), and T\textsubscript{2}b (20 patients). Five patients had lymph node metastases at the time of surgery. This research was approved by the Indiana University Institutional Review Board.

Immunohistochemistry. Serial 5-μm-thick sections prepared from formalin-fixed, paraffin-embedded slices of prostate adenocarcinoma specimens were used for the study. Tissue blocks that contained the maximum amount of tumor and highest Gleason score were selected for each case. One representative slide from each case was analyzed. We recognized the limitation of sample variation. Slides were deparaffinized in xylene twice for 5 minutes and rehydrated through graded ethanol solutions to distilled water. Antigen retrieval was carried out by heating sections in 1 mmol/L EDTA (pH 8.0) for 30 minutes. Endogenous peroxidase activity was inactivated by incubation in 3% H\textsubscript{2}O\textsubscript{2} for 15 minutes. Nonspecific binding sites were blocked using Protein Block (DAKO Corp., Carpinteria, CA) for 20 minutes (30).

Tissue sections were then incubated with the rabbit polyclonal antibody against a recombinant protein corresponding to amino acids 25 to 147 of mature angiogenin I of human origin (IgG 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The specificity of the antibodies was confirmed by immunoprecipitation, Western blot, and ELISA. It should be noted that there is only one form of angiogenin in the human genome (31). After washing with PBS, biotinylated goat anti-rabbit IgG was applied for 30 minutes. Additional washing was done and followed by incubation with peroxidase-labeled streptavidin for 30 minutes. Immunoreactivity was visualized by incubation of section with diaminobenzidine as the chromogen in the presence of hydrogen peroxide. Sections were counterstained with light hematoxylin and coverslip mounted. Negative controls were done using blocking serum in place of primary antibody. A case with known angiogenin expression was used as a positive control. Additionally, normal endothelial cells served as a positive internal control. Positive and negative controls were run in parallel with each series and showed that the procedure functioned properly.

Evaluation of angiogenin expression. The extent and intensity of immunoreactivity for angiogenin were evaluated in benign epithelium, high-grade prostatic intraepithelial neoplasia, and adenocarcinoma from the same slide for each case. Microscopic fields with the highest degree of immunoreactivity were chosen for analysis. At least 1,000 cells were analyzed in each case. The percentage of cells exhibiting staining in each case was evaluated semiquantitatively on a 5% incremental scale ranging from 0% to 95%. A numerical intensity score was set from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). These methods have been previously described (30, 33).

Statistical analysis. The mean percentage of immunoreactive cells in benign epithelium, high-grade prostatic intraepithelial neoplasia, and adenocarcinoma were compared using the Wilcoxon paired signed rank test. The intensities of staining for angiogenin in benign epithelium, high-grade prostatic intraepithelial neoplasia, and adenocarcinoma were compared using Cochran-Mantel-Henszel tests for correlated ordered categorical data. Percentage of immunoreactive cells was compared with clinical and pathologic variables using either Spearman’s rho correlation or one-way ANOVA, if the variable was categorical or continuous, respectively. Because of the large number of variables tested, the false discovery rate method was used to adjust for multiple testing (34). P < 0.05 was considered significant, and all Ps were two sided.

Results

Patient characteristics were illustrated in Table 1. Immunohistochemistry for angiogenin distinguished benign prostatic epithelium from both high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma (Fig. 1). Positive angiogenin immunoreactivity was present in all cases of high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma, whereas in 97% (104 of 107) of cases, benign epithelial cells were either nonreactive or expressed only ≥1 intensity. Significant differences in percentage of cells staining positively for angiogenin separated normal prostatic epithelium from both high-grade prostatic intraepithelial neoplasia (P < 0.001) and prostatic adenocarcinoma (P < 0.001) but did not distinguish high-grade prostatic intraepithelial neoplasia from prostatic adenocarcinoma. Similarly, significant differences in staining intensity existed between benign prostatic epithelium and high-grade prostatic intraepithelial neoplasia (P < 0.001) and between benign prostatic glandular epithelium and prostatic adenocarcinoma (P < 0.001). However, a significant increase in staining intensity was observed between high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma (P < 0.0023).

The distribution of negative, weak, moderate, and strong staining for angiogenin in benign prostatic epithelium was 10% (11 of 107), 87% (93 of 107), 3% (3 of 107), and 0% (0 of 107), respectively. The mean percentage of cells that stained positively for angiogenin expression was 17% in normal epithelium, 58% in high-grade prostatic intraepithelial neoplasia, and 60% in prostatic adenocarcinoma (Table 2).

Eighteen cases contained areas of proliferative inflammatory atrophy (PIA), a putative precursor to prostatic adenocarcinoma (35, 36). These cases exhibited positive staining for angiogenin with intensity ranging from ≥1 to ≥2 with a mean intensity of 1.1. The mean percentage of cells staining positive for angiogenin was 23% with a range of 5% to 50%. No statistically significant difference for expression of angiogenin was observed between PIA and normal benign prostatic epithelium by percentage of cells staining (P = 0.062) and by staining intensity (P = 0.15).
Although positive angiogenin immunoreactivity could segregate benign prostatic epithelium from both high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma, positive angiogenin expression did not correlate with additional clinical and pathologic variables examined. There was no significant correlation between the level of angiogenin expression and other clinicopathologic features, including age at surgery, Gleason scores, pathologic stage, tumor extent, angiolymphatic invasion, extraprostatic extension, seminal vesical invasion, lymph node metastasis, surgical margin status, presence of prostatic intraepithelial neoplasia, and perineural invasion.

**Discussion**

Angiogenin was originally isolated from HT-29 human colon adenocarcinoma cells and has both angiogenic and non-angiogenic properties. In nonneoplastic conditions, angiogenin is a component of the intercellular matrix encircling endothelium, fibroblasts, and smooth muscle as well as the internal elastic membrane of arterioles and capillaries (6). It induces neovascularization using the chick chorioallantoic membrane, meniscus, rat cremaster muscle, and rabbit cornea models (27, 37, 38). Angiogenin expression is regulated by the Akt/phosphatidylinositol 3-kinase pathway (9, 39). Classified as a member of the RISPASE (RNases with special biological actions) family of RNases, angiogenin possesses homology with other RNases, such as pancreatic RNase A. The ability of angiogenin to induce development of new blood vessels has been shown to be dependent on its ribonucleic activity (9, 39). It induces angiogenesis by increasing endothelial degradation of extracellular matrix and basal lamina consequently promoting invasion and migration of individual cells (6). It is hypothesized that angiogenin stimulates the polymerization and release of cell surface actin, a process that may be necessary for eventual cell migration (6, 37, 40–43). A study using cultured endothelium has shown that angiogenin binds to extracellular matrix providing scaffolding for and simultaneously directing proliferating cells (6). It binds to the cell surface of endothelial cells and undergoes nuclear translocation, a process requisite for induction of angiogenesis (27). The definitive receptor for angiogenin has not been identified, although several promising candidates exist. Angiogenin accumulates in the nucleolus and promotes rRNA transcription, ultimately leading to proliferation of the endothelial cells (27). Kishimoto et al. have reported that angiogenin activity is a necessary requirement for endothelial cell proliferation induced by acidic and basic fibroblast growth factors, vascular endothelial growth factor, and

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<th>Table 1. Patient characteristics and angiogenin staining percentage and intensity in prostatic adenocarcinoma</th>
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<td>Patient characteristic</td>
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<tr>
<td>Gleason sum</td>
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<tr>
<td>&lt;7</td>
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<tr>
<td>≥7</td>
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<tr>
<td>Pathologic stage</td>
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<tr>
<td>T2</td>
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<tr>
<td>T3a</td>
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<td>T3b</td>
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<tr>
<td>Tumor diameter (cm)</td>
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<tr>
<td>0.1-1.0</td>
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<tr>
<td>1.1-2.0</td>
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<tr>
<td>&gt;2.0</td>
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<tr>
<td>Extraprostatic extension</td>
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<td>Positive</td>
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<td>Negative</td>
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<td>Surgical margin</td>
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<td>Vascular invasion</td>
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<td>Perineural invasion</td>
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<tr>
<td>Negative</td>
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<tr>
<td>High-grade PIN</td>
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<td>Negative</td>
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Abbreviation: PIN, prostatic intraepithelial neoplasia.
epidermal growth factor. Additionally, these authors showed that disruption of nuclear translocation of angiogenin prevents the angiogenic activity of the aforementioned factors. Indeed rRNA production stimulated by angiogenin may represent a “crossroad” in the pathway of angiogenesis promoted by multiple angiogenic proteins (27).

In this study, we found that angiogenin was expressed in a significantly greater percentage of cells in high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma compared with benign prostatic glandular epithelium. Moreover, the percentage of cells expressing angiogenin did not differ between high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma. Only in benign cases did cells fail to stain for angiogenin, whereas all cases of high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma expressed angiogenin in at least 10% of cells, with mean values of 58% and 60%, respectively. The intensity of staining for angiogenin also differed significantly between benign prostatic epithelium and high-grade prostatic intraepithelial neoplasia and between benign prostatic glandular epithelium and prostatic adenocarcinoma. Additionally, a difference was observed between high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma for intensity of staining. Furthermore, all cases of high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinoma showed at least ≥1 staining intensity, with mean values of 1.37 and 1.70 intensity grades, respectively. PIA is a proposed precursor to prostatic adenocarcinoma (35, 36). In our study, we examined 18 cases with PIA and did not identify an increased expression of angiogenin in PIA when compared with benign prostatic glandular epithelium. However, caution is warranted in interpreting our data, as our sample size is limited.

Our findings of increased expression of angiogenin in prostatic adenocarcinoma may have practical implication for the treatment of prostate cancer. The significant expression of angiogenin in prostatic adenocarcinoma and high-grade prostatic intraepithelial neoplasia coupled with recent elucidation of the inhibitory effects of antiangiogenin agents in animal models indicate that the inhibition of angiogenin may represent a promising target for the treatment of human prostatic adenocarcinoma.

The hypothesis that angiogenic activity is an early requirement for a cell population to become committed to malignancy has been previously proposed (4, 44, 45). Indeed, Folkman and Watson first proposed this concept after examining the progression of pancreatic islet β cells from normal to hyperplasia and ultimately neoplasia (44). Although in our study, both premalignant and malignant cells expressed angiogenin, the intensity of its expression (as measured by immunohistochemical staining intensity) was significantly increased in prostatic adenocarcinoma versus high-grade prostatic intraepithelial neoplasia. This finding may indicate that the quantity of angiogenin expression becomes amplified as the cells proceed to an invasive phenotype.

Tumor angiogenesis occurs by means of a complex mechanism, which balances endothelial cell apoptosis with replication to create an increase in tumor microvascular density (24). The cycling of endothelial cell migration, division, and differentiation results in new capillary formation (6). The principal molecular players in angiogenesis include vascular endothelial growth factor, platelet-derived endothelial cell growth factor, basic fibroblast growth factor, thrombospondin, pleiotrophin, endostatin, and angiogenin. Angiogenesis plays a major role in the proliferation, invasion, and distant spread of malignant neoplasms, including prostatic adenocarcinoma (1, 3). Angiogenesis has been shown to facilitate progression and metastasis in other tumor types, including colon, gastric, head and neck squamous, hepatocellular, pancreatic, prostatic, and urothelial carcinomas; melanoma; and gestational trophoblastic tumors (13–22). Indeed, angiogenesis is felt to be
requisite for tumors to grow beyond 2 mm (46). Increased expression of angiogenin as shown by immunohistochemistry, in situ hybridization, or mRNA expression has been reported in multiple other solid cancers, including colorectal, gastric, hepatocellular, pancreatic, and urothelial carcinomas (7, 14, 16, 19, 47). Moreover, elevated serum angiogenin levels have been documented in numerous cancers, including prostatic adenocarcinoma (7, 15–19, 21, 22). In many cases, the degree of serum angiogenin elevation has been correlated with tumor aggressiveness (13–17, 19). In particular, significantly higher serum levels of angiogenin have been found in men with prostatic adenocarcinoma versus those free of tumor (32). Elevated levels of other angiogenic factors, such as endothelin-1, have also been found in high-grade prostatic intraepithelial neoplasia and in the plasma of men with prostatic adenocarcinoma (32). In a study of angiogenin inhibition by monoclonal antiangiogenin antibody in an animal model of prostate cancer, Olson et al. briefly described overexpression of angiogenin using immunohistochemistry in prostatic adenocarcinoma from 10 patients versus normal prostate tissue from seven different individuals (4). However, statistical analysis of the small sample was not done to assess the differences observed in immunohistochemical expression of angiogenin, and these observations were not correlated with clinicopathologic variables. To our knowledge, our present investigation of 107 cases of prostatic adenocarcinoma is the first large series to examine whether a significant difference in angiogenin expression existed among prostatic adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and benign glandular prostatic epithelium and to correlate the findings with multiple clinicopathologic variables. Furthermore, we believe our study to be the first to examine the expression of angiogenin in PIA and to compare PIA expression of the angiogenin polypeptide with prostatic adenocarcinoma, high-grade prostatic intraepithelial neoplasia, and benign glandular prostatic epithelium.

Our findings reveal that angiogenin is overexpressed as measured by percentage of cells staining and by staining intensity in prostatic adenocarcinoma and high-grade prostatic intraepithelial neoplasia compared with benign prostatic glandular epithelium. The finding that the percentage of cells staining did not significantly increase in the progression from high-grade prostatic intraepithelial neoplasia to prostatic adenocarcinoma may indicate that angiogenin is involved early in the evolution of invasive malignancy and becomes expressed while cells are still in a premalignant stage. One of the limitations of our study was that only one representative section from each case was used for performance of immunohistochemistry. This sampling method may have introduced variation into our results among cases. However, to control for this variation and to give the best representative section we chose blocks, which contained the maximum amount of tumor and contained the highest Gleason score for each case.

In animal studies using athymic mice, Olson et al. showed that inhibition of angiogenin by monoclonal antibody prevented the establishment of androgen-independent human prostate cancers in athymic mice and reduced the formation of spontaneous regional metastasis derived from the primary tumor (4). Antisense-targeted disruption of angiogenin gene expression has been shown to inhibit the development and dissemination of human prostate cancer xenografts in athymic mice (25). In prostatic adenocarcinoma specifically, an increase in blood vessel density, a measurement of angiogenesis, has been correlated with a poor prognosis (23, 24). Our findings in concert with a growing body of research implicate angiogenin as an important mediator in the growth and metastasis of prostatic adenocarcinoma. Agents that inhibit the activity of angiogenin offer promising potential in the treatment of prostatic adenocarcinoma and perhaps many other malignant neoplasms for which angiogenin-mediated angiogenesis plays a critical role. The overexpression of angiogenin in human prostate, coupled with the ability of angiogenin-inhibitory agents to prevent growth and metastasis of human cell line prostatic adenocarcinoma in animal models, make antiangiogenin therapy a promising area for future cancer therapy.

Table 2. Intensity and percentage of cells with angiogenin immunostaining in 107 radical prostatectomy specimens

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<tr>
<th>Cell type</th>
<th>Staining intensity grade (%)</th>
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<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Normal (n = 107)</td>
<td>11 (10)</td>
<td>93 (87)</td>
<td>3 (3)</td>
<td>0 (0)</td>
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<tr>
<td>High-grade PIN* (n = 98)</td>
<td>0 (0)</td>
<td>62 (63)</td>
<td>35 (36)</td>
<td>1 (1)</td>
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<tr>
<td>Adenocarcinoma* (n = 107)</td>
<td>0 (0)</td>
<td>53 (50)</td>
<td>45 (42)</td>
<td>9 (8)</td>
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<tr>
<th></th>
<th>Mean % cells staining ± SD</th>
<th>Median (range), %</th>
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<tr>
<td>Normal (n = 107)</td>
<td>17 ± 15</td>
<td>10 (0–80)</td>
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<tr>
<td>High-grade PIN* (n = 98)</td>
<td>58 ± 22</td>
<td>60 (10–90)</td>
</tr>
<tr>
<td>Adenocarcinoma* (n = 107)</td>
<td>60 ± 24</td>
<td>70 (10–90)</td>
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Abbreviation: PIN, prostatic intraepithelial neoplasia.

*Staining intensity was statistically higher compared with that of the normal cells with P < 0.001 using a Cochran-Mantel-Henszel test.

1Staining intensity was statistically higher compared with high-grade PIN with P = 0.0023 using a Cochran-Mantel-Henszel test.

2Percentage of staining was statistically higher compared with that of the normal cells with P < 0.001 using a Wilcoxon paired signed rank test.
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