SHORT COMMUNICATION

ProNGF Correlates with Gleason Score and Is a Potential Driver of Nerve Infiltration in Prostate Cancer

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Nerve infiltration is essential to prostate cancer progression, but the mechanism by which nerves are attracted to prostate tumors remains unknown. We report that the precursor of nerve growth factor (proNGF) is overexpressed in prostate cancer and involved in the ability of prostate cancer cells to induce axonogenesis. A series of 120 prostate cancer and benign prostate hyperplasia (BPH) samples were analyzed by IHC for proNGF. ProNGF was mainly localized in the cytoplasm of epithelial cells, with marked expression in cancer compared with BPH. Importantly, the proNGF level positively correlated with the Gleason score ($n = 104$, $\tau_B = 0.51$). A higher level of proNGF was observed in tumors with a Gleason score of ≥ 8 compared with a Gleason score of 7 and 6 ($P < 0.001$). *In vitro*, proNGF was detected in LNCaP, DU145, and PC-3 prostate cancer cells and BPH-1 cells but not in RWPE-1 immortalized nontumorigenic prostate epithelial cells or primary normal prostate epithelial cells. Co-culture of PC12 neuronal-like cells or 50B11 neurons with PC-3 cells resulted in neurite outgrowth in neuronal cells that was inhibited by blocking antibodies against proNGF, indicating that prostate cancer cells can induce axonogenesis via secretion of proNGF. These data reveal that ProNGF is a biomarker associated with high-risk prostate cancers and a potential driver of infiltration by nerves. (*Am J Pathol* 2014, 184: 3156–3162; <http://dx.doi.org/10.1016/j.ajpath.2014.08.009>)

A recent study¹ reported that autonomic nerve sprouting in prostate tumors contributes to prostate cancer progression. Sympathetic and parasympathetic nerve fibers were found to be necessary from initial to late phases of prostate cancer development. The density of nerve fibers in human prostate cancers was directly correlated to the Gleason prostate cancer score, and in an animal model, denervation resulted in a decrease in tumor engraftment and metastasis via a mechanism that involved the stimulation of β -adrenergic receptors on the surface of prostate cancer cells. This study is the first clear demonstration that the nervous system is involved in cancer progression and that nerve fibers are an essential component of the tumor microenvironment, participating in cancer growth and metastasis. However, the mechanisms by which nerve fibers are attracted to prostate tumors remain to be elucidated.

Nerve growth factor (NGF), the archetypal neurotrophin, is essential to the development of the peripheral and autonomic

nervous systems because organs targeted by innervation produce NGF to stimulate neurite outgrowth and attract nerve fibers.² NGF drives the progressive innervation of the body and acts on neurons through the membrane tyrosine kinase receptor TrkA and the death receptor p75^{NTR}, activating various signaling pathways, including the mitogen-activated protein kinases, phosphatidylinositol 3-kinase, and NF- κ B. The production of NGF is made through the synthesis of a biochemical precursor, proNGF, which can be cleaved either intracellularly by the protease furin or extracellularly by

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metalloproteases to produce mature NGF.³ Thus, proNGF is a reservoir of mature NGF, and the level of proNGF in a tissue reflects *NGF* gene expression. Interestingly, proNGF per se is also a biologically active molecule, initially described as an inducer of neuron apoptosis through interaction with sortilin and p75^{NTR}.⁴ However, recent studies have also described its neurotrophic activities (ie, its ability to induce axonogenesis), and proNGF can stimulate neuron survival and differentiation by direct interaction with TrkA and p75^{NTR}, resulting in the activation of the mitogen-activated protein kinase pathway.^{5–9}

In prostate cancer cells, a high-molecular-weight anti-NGF reactive protein, presumably corresponding to proNGF, has been described.¹⁰ Therefore, by analogy with embryonic and postnatal development, it could be hypothesized that proNGF expression participates in attracting nerve fibers in prostate cancers. However, the production of proNGF has not been reported in a large cohort of prostate cancers, and it is not known whether it correlates with cancer aggressiveness. We explored the level of proNGF in such a cohort of prostate cancers and investigated a possible correlation with Gleason score. In addition, we co-cultured prostate cancer cells with neuronal cells to test the hypothesis that proNGF production by prostate cancer cells could stimulate axonogenesis.

Material and Methods

Prostate Tissue Samples and Cell Lines

High-density tumor microarrays of prostate cancers and adjacent benign prostatic hyperplasia (BPH) (PR802 and PR951) were obtained from Biomax (Rockville, MD). This cohort contained a large proportion of high-grade prostatic cancers, and information on treatment of patients was not available. Primary prostate epithelial cells (PrECs) were purchased from Lonza (Walkersville, MD). LNCaP cells (extracted from a lymph node metastasis of prostate cancer) were purchased from the ATCC (Manassas, VA) (CRL-1740). PC-3 prostate cancer cells (ATCC CRL-1435, derived from a bone metastasis of prostate cancer), BPH-1 cells (from a patient with BPH), DU145 cells (ATCC HTB-81, derived from a brain metastasis of prostate cancer), and RWPE-1 transformed nontumorigenic prostate epithelial cells were gifts from Dr. Judith Weidenhofer (University of Newcastle, NSW, Australia). The neuronal-like PC12 cells (ATCC CRL-1721) were from Prof. Ralph A. Bradshaw (University of California, San Francisco, CA). Immortalized dorsal root ganglia neurons 50B11 were obtained from Dr. Ahmet Höke (John Hopkins University, Baltimore, MD). PrECs were cultured in prostate primary epithelial cell culture media supplemented with PrEGM SingleQuots (Lonza, Walkersville, MD). BPH-1, RWPE-1, LNCaP, PC-3, and DU145 cells were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum (JRH Biosciences, Lenexa, KS). PC12 cells were maintained in Dulbecco's modified eagle medium from Life

Technologies (Victoria, Australia) with 5% fetal calf serum, 10% horse serum (Sigma, South Australia, Australia), and 2 mmol/L L-glutamine. 50B11 neurons were maintained in neurobasal-A medium (Invitrogen, Victoria, Australia) with 10% fetal calf serum. All cell lines were grown in 75 cm² tissue culture flasks in a humidified incubator at 37°C with 5% CO₂.

Immunohistochemistry

After deparaffinization and hydration of tumor microarrays, antigens were retrieved at 60°C in 10 mmol/L citrate (pH 6) buffer. Endogenous peroxidases were quenched by immersing slides in TBS-Tween 0.1%, containing 3% H₂O₂ (10 minutes at room temperature), and saturation in Tris-buffered saline and Tween 0.1% with 3% bovine serum albumin (60 minutes, 37°C) was performed. Slides were probed with anti-proNGF antibody (AB9040 from Millipore, Billerica, MA) or normal rabbit IgG (AB-105-C from R&D Systems, Minneapolis, MN) as a negative control, at 1/200 in saturating buffer for 2 hours at 37°C. The signal was amplified with horseradish peroxidase–conjugated antibodies 711-035-152 anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) 1/400 in saturating buffer for 2 hours at 37°C. Immunostaining was visualized with diaminobenzidine chromogen (Sigma-Aldrich, St. Louis, MO), and slides were poststained with Harris hematoxylin and mounted before observation with a Nikon Eclipse Ti-U microscope.

Double Immunostaining

Stainings were performed sequentially using ImmPRESS reagents as per the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Briefly, after deparaffinization, rehydration, inactivation of endogenous peroxidases with H₂O₂, and blocking with 2.5% horse serum, the mouse proNGF was first applied to the sections followed by the ImmPRESS horseradish peroxidase anti-mouse IgG (peroxidase) and incubation with diaminobenzidine peroxidase substrate solution (Vector Laboratories). Then the slides were washed for 5 minutes in phosphate-buffered saline, blocked, stained with rabbit PGP9.5 (Abcam, Cambridge, MA) antibody, and incubated with the ImmPRESS alkaline phosphatase anti-rabbit IgG before incubation with Vector Red alkaline phosphatase substrate (Vector Laboratories). Finally, tissue microarray slides were counterstained with hematoxylin ([Supplemental Figure S1](#)).

Quantification of proNGF Labeling and Analysis of Correlation with Gleason Score

Determination of prostate tumor Gleason score according to the International Society of Urological Pathology 2005 modified Gleason grading system¹¹ was performed by a histopathologist (M.M.W.). Tissues used in the present study all had architectural patterns considered to be Gleason

grade 3, 4, or 5 and were categorized as a Gleason score of 6 (3+3), a Gleason score of 7 (3+4 and 4+3), and a Gleason score of ≥ 8 (4+4, 4+5, 5+4, 5+5). Two independent observers estimated the intensity of anti-proNGF staining as 0 (no staining), 1 (weak staining), 2 (medium staining), and 3 (intense staining), as described previously.¹² For association of proNGF staining intensity with Gleason grade (3, 4, or 5), a total of 264 tissue areas from 120 patients (104 cancers + 16 cases of BPH) were available for analysis. Individual Gleason grade and overall Gleason score (the sum of the two most represented Gleason grade areas per patient) was compared with proNGF staining intensity and analyzed as ordinal variables using χ^2 or Fisher's exact tests, with Kendall's τ_B rank correlation coefficient calculated as a measure of association. Analysis was performed using Statistica statistical software version 10 (StatSoft Inc., Tulsa, OK), and STATA statistical software version 11 (StataCorp, College Station, TX).

Digital Analysis of proNGF Labeling

Ten representative regions of interest were defined for each image, highlighting epithelial areas. Diaminobenzidine staining was revealed using the Color Deconvolution algorithm (version 9.1) before applying the Positive Pixel Count algorithm (version 9.1) to obtain the mean intensity of all pixels in each region of interest. Nuclei in each region of interest were counted, and a cell density index was calculated as number of cells per square millimeter. Box and whisker plots were generated from the data using BoxPlotR software (interactive web application running in R version 3.1 using Shiny version 1.2) ([Supplemental Figure S2](#)).

Protein Extraction and Western Blotting

Subconfluent cell monolayers were lysed with 1% NP40 lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40 pH 8.0) that contained complete EDTA free protease inhibitor cocktail (Roche, Mannheim, Germany). Insoluble proteins were removed by centrifugation at $15 \times 10^3 \times g$ for 10 minutes (at 4°C), and the total protein concentration was determined using the micro BCA kit (Pierce Biotechnology, Rockford, IL) per the manufacturer's instructions. Next 20- μ g proteins were separated by SDS-PAGE with 12% resolving gel and then transferred to 0.4- μ m pore nitrocellulose membranes (Amersham, GE Healthcare Life Sciences, Pittsburgh, PA) using a wet transblotter (BioRad, Gladesville, NSW, Australia). Blots were blocked with blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature and then probed with antibodies against proNGF (1/500; AB9040 from Millipore) and β -actin (1/5000; Sigma-Aldrich) diluted in the blocking buffer. After washing with phosphate-buffered saline that contained 0.1% Tween 20, membranes were probed with goat anti-mouse or goat anti-rabbit IR-Dye 670 or 800cw-labeled secondary antisera then washes were

repeated after labeling. Western blot was imaged using the LI-COR Odyssey infrared imaging system (LI-COR) and densitometric analysis of immunoreactive bands was determined using the Image Studio Lite (LI-COR).

Co-Cultures and Neurite Outgrowth Assay

For co-culture experiments, PC-12 cells (5×10^4 in 1 mL) were seeded in the lower compartment of Transwell plates (Corning Inc., Midland, MI) coated with rattail collagen I (Invitrogen). After 24 hours, cells were serum starved in Dulbecco's modified eagle medium that contained 1% horse serum. Co-cultures were performed with PC-3 prostate cancer cells grown in the upper Transwell inserts (12.0 mm in diameter with 3.0- μ m pores) (Corning Inc.) with or without anti-proNGF blocking monoclonal antibodies (Alomone, Jerusalem, Israel). Control was performed with isotype antibody. Differentiation of PC12 cells was allowed for 4 days, and neurite outgrowth was measured per square millimeter. PC12 cells were considered differentiated when they exhibited neurites of at least twice the length of the cell body. For co-culture with the 50B11 cells, the same protocol was applied, but the culture media included 5 μ mol/L

Figure 1 Immunohistochemical detection of precursor of nerve growth factor (proNGF). ProNGF was immunodetected in a series of 120 prostate cancers and benign prostate hyperplasia. Representative images are shown. **A and B:** Benign prostate hyperplasia (no staining in epithelial cells, 0). **C:** Gleason score 3+3 cancer (low intensity staining, 1). **D:** Gleason score 4+3 cancer (high intensity staining, 3). **E:** Gleason score 4+5 cancer (high intensity staining, 3). **F:** Gleason score 4+4 cancer (medium intensity staining, 2). **Boxed areas** show higher magnification of proNGF cellular staining. Quantitative image analyses of the tumors presented here are shown in [Supplemental Figure S2](#). Prostate cancers and benign prostate hyperplasia ($n = 120$) were analyzed. Original magnification: $\times 100$ (A–F); $\times 400$ (boxed areas, A–F).

forskolin (necessary to obtain neurite outgrowth with these cells). Images were obtained using an inverted microscope (Zeiss, Jena, Germany). One-way analysis of variance statistical test (GraphPad Prism version 5.01; GraphPad Software, La Jolla, CA) was used.

Results

ProNGF Level Correlates with Gleason Score

Immunohistochemistry (IHC) of cancer tissue samples revealed the presence of proNGF mainly in the cytoplasm of epithelial cancer cells, with stromal cells exhibiting a low level of staining (Figure 1). Absence of proNGF staining was observed in 13% of BPH tissues (Figure 1, A and B), whereas various levels of proNGF could be observed among cancers of different Gleason scores (Figure 1, C–F). Digital

Figure 2 Frequency distribution of precursor of nerve growth factor (proNGF) level according to Gleason score. ProNGF levels [0, no staining (white); 1, low intensity staining (light gray); 2, intermediate intensity staining (dark gray); and 3, high intensity staining (black)] were measured in epithelial cells of benign prostatic hyperplasia (BPH) and prostate cancers. **A:** Distribution according to Gleason score. Tumors with high Gleason scores of 7 and ≥ 8 (3+4, 4+3, 4+5, 5+4, 5+5) were more likely to have high proNGF expression than tumors with low Gleason scores (Gleason grades 3+3) and BPH. The calculated coefficient of correlation between proNGF staining intensity and Gleason score was $r_B = 0.51$. **B:** Distribution according to Gleason grade. The proportion of tissue areas expressing high levels of ProNGF increased in Gleason grade 4 and 5 areas compared with Gleason grade 3 areas. $***P < 0.001$, Fisher's exact test; $****P < 0.0005$, χ^2 test.

Figure 3 Precursor of nerve growth factor (proNGF) production in prostate cancer cell lines. **A:** Western blot detects proNGF in normal prostate epithelial cells (PrECs), transformed nontumorigenic prostate epithelial cells (RWPE-1), benign prostate hyperplasia cells (BPH-1), and prostate cancer cells (LNCaP, DU145, PC-3). A major band is observed at 60 kDa and two other minor bands at 50 and 85 kDa. **B:** Densitometric quantification of proNGF. Normalization was performed relative to β -actin.

quantification (Supplemental Figure S2) indicated an increase in proNGF (regardless of cell density) in Figure 1, C–F (cancer), compared with Figure 1, A and B (BPH). This increase of proNGF on a per cell basis is also visible in enlargement boxes for each panel. The frequency distribution of proNGF levels (Figure 2A) revealed that most BPH and Gleason score 6 cancers had low levels of proNGF (staining intensity, 0 and 1), whereas the proportion of cases with intermediate (staining intensity, 2) and high (staining intensity, 3) levels of proNGF increased in Gleason score 7 and ≥ 8 cancers ($P < 0.001$). Therefore, most cases with the highest Gleason score exhibited the most intense proNGF labeling levels. Highest levels of proNGF (staining intensity, 3) were observed only in Gleason score 7 and ≥ 8 cancers and not in Gleason score 6 cancers. We also looked at proNGF level in individual Gleason grade areas (Gleason score being the sum of the two most abundant Gleason grades observed in a section) (Figure 2B). The proportion of cases with intermediate (staining intensity, 2) and high (staining intensity, 3) levels of proNGF increased in Gleason grade areas of 4 and 5 compared with Gleason grade 3 areas ($P < 0.0005$). Together, there was a positive correlation between proNGF

Figure 4 Axonogenesis is induced by prostate cancer cells. **A:** Co-culture experiments were performed in Transwell Boyden chambers with PC-3 prostate cancer cells in the upper chamber and neuronal cells (PC12 or 50B11) in the lower chamber. Neurite outgrowth started after 3 days. **B** and **C:** After 4 days of co-culture, PC12 and 50B11 cells remain undifferentiated in the absence of PC-3 cells (control), and neurite outgrowth is observed in co-culture with PC-3 (+PC-3). This neurotrophic effect is diminished in the presence of anti-proNGF blocking antibodies (+PC-3 + anti-proNGF antibody), whereas the isotype control antibodies (+PC-3 + isotype antibody) do not inhibit axonogenesis. **Arrows** indicate neurites. **D** and **E:** Quantification of neurite outgrowth in PC12 and 50B11 cells. Experiments were performed in triplicate and data represent means $\pm$ SD. Analysis of variance test was used. * $P < 0.05$, ** $P < 0.01$.

intensity levels and Gleason grades ($n = 264$, coefficient of correlation $\tau_B = 0.37$) that became more pronounced when proNGF intensity levels were considered against Gleason scores ($n = 104$, coefficient of correlation $\tau_B = 0.51$). This pattern of proNGF relative to Gleason score was similar to the nerve infiltration described by Magnon et al¹ because they found that nerve infiltration correlated with Gleason score. Although we have not investigated nerve infiltration in the present study, we have been able to co-localize proNGF with

nerve fibers (Supplemental Figure S1). Together, these data prompted us to look for a proNGF-mediated ability of prostate cancer cells to stimulate axonogenesis.

ProNGF Production in Prostate Cancer Cell Lines

Western blot analysis of a panel of normal and cancer prostate cells indicated a major immunoreactive band at 60 kDa (Figure 3A). On the basis of amino acid sequence, the

theoretical molecular mass of proNGF is 26 kDa, but there are two glycosylation sites, and various apparent molecular masses up to 100 kDa have been reported, including 60 kDa in the uterus.¹³ Interestingly, Djakiew et al¹⁴ used Western blotting with an anti-NGF antibody in prostatic cancer to describe an immunoreactive band of approximately 60 kDa that they called the NGF-like protein. This study was performed before the discovery of proNGF, and our results, confirming the molecular weight of 60 kDa, indicate that it was actually proNGF. Densitometric analysis of the Western blotting (Figure 3B) revealed that proNGF level was minimal in primary PrECs and RWPE-1 immortalized nontumorigenic epithelial cells, increased in the BPH-1 benign prostate hyperplasia cells, and higher in LNCaP, DU145, and PC-3 cancer cells. These data mirror the IHC analyses (Figures 1 and 2) indicating a higher level of proNGF in prostatic cancer cells compared with those from BPH. In addition, mature NGF was not detected in any of the prostate cells tested (data not shown), suggesting that proNGF is not processed to mature NGF in prostate cancer cells.

Neurotrophic Activity of Prostate Cancer Cells Involves proNGF

Co-culture experiments in Transwell Boyden chambers were performed between PC-3 prostate cancer cells and PC12 neuronal-like cells or the 50B11 immortalized dorsal root ganglia neurons (Figure 4A). The results revealed that PC-3 cells could induce neuronal differentiation of PC12 cells (Figure 4B) and 50B11 cells (Figure 4C). Neurite outgrowth was observed when PC12 cells or 50B11 cells were co-cultured with PC-3, whereas no differentiation occurred in the control situation in which the neuronal cells were grown alone. Interestingly, blocking antibodies against proNGF diminished PC-3–induced differentiation of PC12 and 50B11 cells (Figure 4, B and C). Less differentiated cells and shorter neurites were observed in the presence of the anti-proNGF antibodies. The number of differentiated cells was quantified for PC12 and 50B11 (Figures 4D and 4E). Together these data reveal that prostate cancer cells are able to induce axonogenesis through the production of proNGF.

Discussion

The level of proNGF that we describe here in relation to Gleason score in prostate tumors is comparable to the association between Gleason score and the density of nerve infiltration reported by Magnon et al.¹ They found a higher density of nerve fibers in cancers of high Gleason score. However, they did not suggest an explanation for the mechanism of nerve infiltration. Although we have no human data on axonogenesis, our observation that proNGF is increased in cancers of high Gleason score points to proNGF overexpression by prostate cancer cells as a possible explanation for nerve infiltration in prostate cancer. By using co-culture with PC12 cells and 50B11 neurons, we found that prostate cancer cells can induce neuronal differentiation and axonogenesis by a

mechanism that involves the production of proNGF. PC12 cells are not true neurons, but they are the canonical *in vitro* model for studying neurotrophic factors and their signaling pathways. Membrane receptors for NGF and proNGF, TrkA-p75^{NTR}-sortilin are expressed in PC12 cells and mediate NGF-stimulated induction of neurodifferentiation. It has been found that proNGF can induce differentiation of PC12 cells even though it is unclear whether proNGF acts directly or through the generation of mature NGF because it depends on the relative levels of its receptors TrkA and p75^{NTR} and the intracellular cleavage of proNGF into mature NGF.^{8,15} In the present work, NGF could not be detected in prostate cancer cells and conditioned media (data not shown), which therefore suggested that proNGF itself was responsible for axonogenesis. Overall, these data indicated that the production of proNGF by cancer cells is a potential driver of nerve infiltration in prostate cancer.

Prostatic cancer cells have been described to respond to NGF stimulation by increasing proliferation and migration via the activation of the membrane receptors TrkA and p75^{NTR},¹⁶ and changes in the expression of these ligands/receptors contribute to prostate tumor cell growth and dissemination through mitogen-activated protein kinase–regulated signaling pathways.^{17,18} In addition, inhibition of the Trk receptor axis decreases the growth of prostatic cancer cell xenografts in nude mice,¹⁹ indicating that the proNGF/NGF/receptors axis is active in prostate cancer. Our results reveal that a neurotrophic activity of proNGF, produced by prostate cancer cells, is also to be taken into consideration for its effect on tumor progression. From a therapeutic perspective, the study by Magnon et al¹ found that targeting nerve fibers in prostate cancer can inhibit cancer growth and metastasis. However, the drug used (6-hydroxydopamine) is unlikely to be of clinical use to treat prostate cancer because it crosses the blood brain barrier and is highly toxic for the central nervous system.²⁰ Therefore, identifying the cause of nerve infiltration in prostate cancer and finding ways to block nerve infiltration without inducing neuronal toxicity is of great importance to any future translation to the clinic. The identification of proNGF as a driver of prostate cancer innervation offers a rationale for testing the therapeutic potential of targeting this polypeptide growth factor in prostate cancer.

NGF is a mediator of pain, and its receptors TrkA and p75^{NTR} are nociceptors whose activation in sensory neurons results in the transmission of the feeling of pain to the central nervous system.^{21,22} Interestingly, it has been found in a murine model that anti-NGF antibody (which can also target proNGF) decreases pain induced by bone metastasis of prostate cancer cells,^{23,24} and a humanized monoclonal antibody (Tanezumab) has already entered clinical trials for its analgesic activity in chronic and acute pain.²⁵ Therefore, targeting proNGF could also have an additional positive effect in prostate cancer by reducing metastasis-induced pain.

In conclusion, we found that proNGF is overexpressed in human prostate cancer, it correlates with Gleason score, and

its production by cancer cells can potentially drive axonogenesis. Thus, the value of proNGF as a clinical biomarker for prognosis and as a therapeutic target to inhibit nerve infiltration in prostatic cancer should be further investigated.

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Y.D. performed the IHC; tissue slide analysis, grading, and scoring were performed by M.M.W. and confirmed by H.H. and R.T.; J.P. performed the *in vitro* experiments and prepared all figures; P.J. contributed to neuronal cell cultures; L.L. performed statistical analyses; D.B. contributed to cell culture and protein extraction; H.H. conceived and supervised the study with significant input from S.R. and R.A.B.; H.H. and J.P. drafted the manuscript, which was read and approved by all co-authors.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.08.009>.

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