

Aberrant Expression of Chromogranin A, miR-146a, and miR-146b-5p in Prostate Structures with Focally Disrupted Basal Cell Layers: An Early Sign of Invasion and Hormone-refractory Cancer?

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Abstract. Our recent studies have suggested that prostate tumor invasion is triggered by autoimmunoreactions induced focal basal cell layer disruptions (FBCLD) that selectively favor monoclonal proliferation of the overlying progenitors or of a biologically more aggressive cell clone. As circulating chromogranin-A (CgA) levels are found to correlate with tumor progression and the status of hormone refractoriness, our current study attempted to assess whether CgA-positive cells would be preferentially distributed in epithelial structures with FBCLD. Paraffin-embedded specimens from 50 patients with organ-confined prostate cancer were subjected to double immunohistochemical analysis with monoclonal antibodies to basal cells and CgA. From each case, 3-5 randomly selected fields were digitally photographed and the photos were magnified 400% and the numbers of CgA-positive cells in epithelial structures with non-disrupted, focally disrupted, and lost basal cell layer were separately counted. The averaged number of cell for each category was statistically compared with the Pearson's Chi-square test. In addition, morphologically similar structures with and without CgA-positive cell clusters were microdissected from four selected cases and subjected to a comparison of differential micro-RNA expression levels. Our

study revealed that, although isolated CgA-positive cells were seen in both the basal cell layer and the luminal cell population in all cases, only 8 cases (16%) harbored large clusters of CgA-positive cells that were concentrated in a given area, in which all or nearly all cells appeared to share a similar morphological and immunohistochemical profile. Microdissected epithelial structures with CgA-positive cell clusters exhibited a more than 5- and 7-fold lower expression of miR-146a and miR-146b-5p than their CgA-negative counterparts. As focal basal cell layer disruptions and the reduction or loss of miR-146a and miR-146b-5p has been documented to correlate with prostate tumor invasion and hormone refractoriness, our findings suggest that aberrant CgA expression in epithelial structures with FBCLD may represent an early sign of these events.

The normal prostate luminal cell population, which is the histological origin of a vast majority of prostate malignancies, is physically segregated from the stroma by both the basal cell layer and the basement membrane. Basal cells are joined by intercellular junctions and adhesion molecules, forming a continuous sheet encircling the ducts and acini (1, 2). The basement membrane is composed of laminins, type IV collagen, and other molecules, forming a continuous lining surrounding and attaching to the basal cell layer (3, 4). The epithelium is normally devoid of blood vessels and lymphatic ducts, and is therefore dependent upon the stroma for its normal functions and survival. Due to these structural relationships, nutrients and growth factors must first pass through the basement membrane, then the basal cell layer in order to reach the luminal cells. In contrast, luminal cells must first pass through the basal cell layer, then the basement membrane in order to invade or to metastasize.

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Prompted by the fact that the basal cell layer is the sole source of tumor suppressor p63 and maspin (5-8), and that degradation of basal cell layers is a pre-requisite for tumor invasion, our recent studies have attempted to identify early signs of basal cell degradation. Our initial study examined the physical integrity of basal cell layers in 50 patients with co-existing pre-invasive and invasive prostate tumors. Of 2,047 ducts and acini examined, 197 were found to harbor focal disruptions (the absence of the basal cells resulting in a gap larger than the combined size of at least 3 basal cells in at least 3 serial sections) in the basal cell layers. The frequency of focal basal cell layer disruptions ranged from none in 22 cases to over one third of the ducts or acini in 17 cases (9). Compared to their non-disrupted counterparts, focally disrupted basal cell layers had a significantly lower frequency of tumor suppressor expression and proliferation, but a significantly higher rate of degeneration and leukocyte infiltration (9). In contrast, epithelial cells overlying focally disrupted basal cell layers had a significantly higher rate of proliferation and expression of cell growth-, tumor invasion, and stem cell-related markers, including leukemia inhibitory factor (LIF), myeloid cell leukemia 1 (MCL1), a tyrosine kinase receptor protein (KIT), and nuclear receptor co-repressor 2 (NCOR2) (9-12). Based on these and other findings, we have proposed that prostate tumor invasion is triggered by autoimmunoreactions induced focal basal cell layer disruptions that selectively favor monoclonal proliferation of the overlying progenitors or of a biologically more aggressive cell clone (9-12). Our findings and speculations are consistent with a number of recent reports which have consistently shown that tumor cell 'budding' from focally disrupted tumor capsule represents a strong and reproducible prognostic marker predicting prognosis and survival of patients with a variety of human cancer types, including esophageal and colorectal cancer (13-15). Thus, early detection and intervention of cell clusters overlying focally disrupted basal cell layers may have significant scientific and therapeutic value.

Recent studies have revealed that neuroendocrine differentiation of prostate tumors parallels the progression to a hormone-refractory state, and that circulating chromogranin-A (CgA) levels reflect neuroendocrine differentiation and correlate with the state of hormone refractoriness (16-23). Thus, our current study attempted to assess the potential correlation between the distribution of CgA expressing cells and the formation of focally disrupted basal cell layers. The hypothesis tested was that CgA represents an early cell differentiation or tumor stem cell-like marker, and therefore, is preferentially expressed in prostate structures with focally disrupted basal cell layers. Since increasing evidence suggests that miRNAs play an important role in tumor progression and invasion (24), we attempted to decipher the roles of miRNAs in early cancer detection by assessing miRNA expression profile between CgA-positive cells and their morphologically similar CgA-negative counterparts.

Materials and Methods

Formalin-fixed and paraffin-embedded tissue sections from 50 human prostate tumors with both pre-invasive and invasive components were selected for our current study. The tissue blocks or unstained tissue sections were obtained from an antibody testing service and collaborative research projects with the National Cancer Institute, Toms Jefferson University Medical School, Fairfax Hospital, Howard University Hospital, and Beijing 301 Hospital, when the corresponding author (Dr. Yan-gao Man) of the current study served as the Director of Gynecologic and Breast Research Laboratory of the Armed Forces Institute of Pathology.

To identify focally disrupted basal cell layers, sections were subjected to immunohistochemistry with a basal cell phenotypic marker, cytokeratin (CK) 34 β E12 (clone: M0630; Dako, Carpinteria, CA, USA) at a 1:50 dilution according to the manufacturer's protocol. Immunostained sections were examined independently by two investigators. A disruption was defined as the focal absence of basal cells resulting in a gap larger than the combined size of at least three basal cells in at least three immediate adjacent sections.

To identify the physical distribution of CgA-positive cells in epithelial structures with and without basal cell layer disruptions, sections were double immunostained with a monoclonal antibody specifically towards human CgA (clone: SP12; NeoMarkers, Fremont, CA, USA) and CK 34 β E12. From each case, 3-5 randomly selected fields at $\times 20$ were digitally photographed and the photos were viewed under a magnification of 400%. The absolute numbers of CgA-positive cells in epithelial structures with non-disrupted, focally disrupted, and lost basal cell layer were separately counted. The number in each category of each case was added and averaged, and the averaged number of each category was statistically compared with the Pearson's Chi-square test. Statistical significance was defined as $p < 0.05$.

To assess whether CgA expression is potentially associated with higher cell proliferation rate, an immediate adjacent section from each case was double immunostained for CK 34 β E12 and a cell proliferation marker, Ki-67. The cell proliferation indices in epithelial structures with and without focally disrupted basal cell layers were statistically compared with the Pearson's Chi-square test. Statistical significance was defined as $p < 0.05$.

Immunostaining was carried out using our published protocol (25). The secondary antibody, ABC detection kit, and diaminobenzidine (DAB) chromogen kit were obtained from Vector (Burlingame, CA, USA). The AP red-chromogen kit was purchased from Zymad (South San Francisco, CA, USA). To assess the specificity of the immunostaining, different negative controls were used, including (i) the substitution of the primary antibody with normal serum, (ii) the omission of the secondary antibody, and (iii) serial dilutions of the primary antibody. In addition, the immunostaining procedure was repeated at least twice using the same protocol and under the same conditions. Immunostained sections were independently evaluated by two investigators. A given cell was considered immunoreactive if distinct immunoreactivity was consistently seen in its cytoplasm, membrane, or nucleus, while all negative controls lacked distinct immunostaining.

Four cases with larger clusters of CgA-positive cells were selected for molecular assays. From each case, 5 unstained sections were deparaffinized and very lightly stained with hematoxylin. Using H&E and immunohistochemically stained sections as references, epithelial structures with basal cell layer disruptions along with multiple CgA-positive cells and their

adjacent morphologically similar counterparts with no CgA-negative cells (as example shown in Figure 2) were separately microdissected for isolation of total RNAs using a published protocol (26). The quantity of RNA was measured by Nanodrop (26), and the quality was assessed by an Agilent 2100 Bioanalyzer using the RNA 6000 LabChip kit (26), measuring the RNA integrity number (RIN). miRNA expression profiling was performed on an Agilent Human microRNA Microarray V3 Technology platform according to the manufacturer's protocols. In brief, 100 ng of total RNA was dephosphorylated by phosphatase at 37°C for 30 min. Then 2.8 µl of 100% dimethyl sulfoxide (DMSO) was added to the dephosphorylated RNA before incubating at 100°C for 7 min, and then immediately cooling in an ice-water bath. The labeling agents were added and incubated at 16°C for 2 h, followed by drying in a speed vacuum at 55°C for 3 h. The hybridization mixtures were then added and incubated at 100°C for 5 min before incubating in an ice-water bath for 5 min. Samples were then loaded onto the microarray slides and hybridized at 55°C for 20 h, with 20 rpm rotating. After hybridization, slides were washed in GE Wash Buffer (Agilent). The slides were scanned using an Agilent High-Resolution C Scanner, and the data were collected and extracted by the Agilent Feature Extraction Software 9.05. All the raw data from Feature Extraction were logarithmic transformed on base 2, quartile algorithm normalized, and brought baseline to median as described elsewhere (27). Student's *t*-test analysis module integrated in Genespring GX11.0 (27) was used to identify miRNAs differentially expressed between CgA-positive and -negative groups, with a *p*-value <0.05, and fold change ≥1.5.

Taqman miRNA quantitative real-time PCR was used to verify the microarray data. RT reaction mixture included 10 ng of total RNA, 3 µl of 5× RT primer, 1.5 µl of 10× RT buffer, 0.15 µl of 100 mM dNTPs, 1 µl of MultiScribe reverse transcriptase, 0.19 µl of RNase inhibitor, and 4.16 µl of nuclease-free water. The 15 µl total reaction mixture was incubated on an ABI 2720 thermal cycler (27) for 30 min at 60°C, 30 min at 42°C, and 5 min at 85°C and then held at 4°C. Real-time qPCR was performed on an ABI 7300 real-time PCR system (28). The 1.5 µl of 1:1 diluted RT product, 10 µl of Taqman Universal PCR Master mixture with no AmpErase UNG, 7.5 of nuclease-free water and 1 µl of 20× miRNA were mixed in a 8-well optical stripe tubes, and then incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All assays were carried out in duplicate with nuclease-free water as a no template control. Relative miRNA expression was compared by calculating 2^{-ΔΔCT} method (28, 29). To normalize the qPCR, U6 snRNA was chosen as the reference gene due to its consistent expression across all samples.

To assess whether differentially expressed miRNAs in CgA-positive cells play a role in prostate cancer, we used three commonly used miRNA target prediction databases: TargetScan 5.1 (30), Diana Micro T3.0 (31), and miRanda (32), which were established on three different algorithms to gauge the potential miRNA targets. Putative miRNAs were considered valid only if they were predicted by at least two programs. We also performed Ingenuity Pathway Analysis (IPA) to assess how those predicted targets are relevant to cancer progression and invasion.

Results

Distinct immunoreactivity to CK βE12 was exclusively seen in morphologically distinct basal cells. Distinct immunoreactivity to CgA was seen in both the basal and

Table I. *MicroRNAs deregulated between CgA-positive and -negative counterparts.*

System ID	<i>p</i> -Value	Regulation status	Fold change
Has-miR-146a	0.02013	Down-regulation	7.396072
Has-miR-146b-5p	0.04747	Down-regulation	5.551642

luminal cells in each case (Figure 1). All negative controls completely lacked distinct immunoreactivity to either of the markers used.

The numbers of CgA-positive cells varied substantially among cases, and 8 cases harbored large clusters of CgA-positive cells that were concentrated in a given area, in which all or nearly all cells appeared to share a similar morphological and immunohistochemical profile (Figure 2). CgA-positive cells were predominantly distributed in epithelial structures with focally disrupted basal cell layers. Of a total of 1,102 CgA-positive cells counted, 793, 94, and 215 were associated with epithelial structures demonstrating focally disrupted, non-disrupted, and lost basal cell layers, respectively. The differences in CgA-positive cells among these structures are statistically significant (*p*<0.01). Epithelial structures with clusters of CgA-positive cells showed a substantially higher cell proliferation and leukocyte infiltration compared to their morphologically similar counterparts with no or fewer CgA-positive cells (data not shown).

Student's *t*-test between CgA-positive and CgA-negative samples revealed two significant deregulated microRNAs, miR-146a and miR-146b-5p, with *p*-value<0.05 and fold change >7 and >5, respectively (Figure 3; Table I). Although miR-146a and miR-146b-5p have different precursor miRNAs (pre-miRNA) located at different chromosomes, they share great similarity in their nucleotide sequences except for several bases after modification and excision from the stem-loop, suggesting similar functionality between them.

Potential miRNA targets were predicted by all three miRNA target prediction databases. IPA analysis revealed that the top five diseases/disorders caused by aberrant expression of miR-146a and miR-146b-5p are cancer, gastrointestinal disease, genetic disorder, inflammatory disease, and infectious disease. The five most significant canonical pathways involved are regulation of interleukin-2 (IL2) expression in activated and anergic T lymphocytes, notch signaling, estrogen receptor signaling, methionine metabolism, and the activation of the peroxisome proliferator-activated receptors (PPAR) and the retinoid X receptors (RXR).

Discussion

It is a commonly held belief that prostate tumor invasion or metastasis is a multistage process, progressing sequentially from normal, to hyperplasia, to prostatic intraepithelial

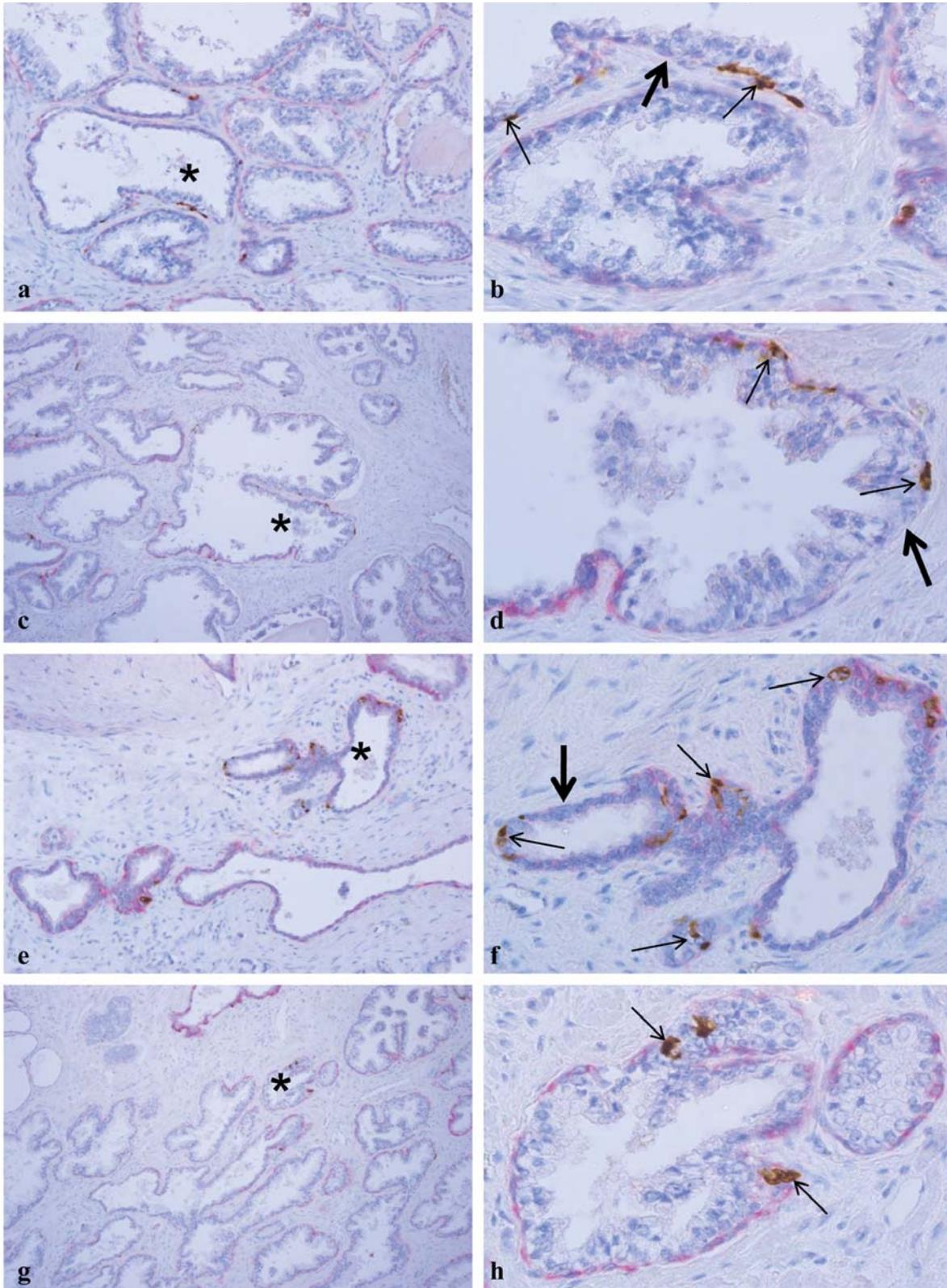


Figure 1. Isolated CgA-positive cells in the prostate basal cell layer and luminal cell population. Paraffin-embedded human prostate tissue sections were double immunostained for CKβE12 (red) and CgA (brown). Thin arrows identify CgA-positive basal or luminal cells. Thick arrows identify focally disrupted basal cell layers. a, c, e, and g: $\times 100$; b, d, f, and h: higher magnification ($\times 400$) of areas marked with an asterisk in a, c, e, and g, respectively.

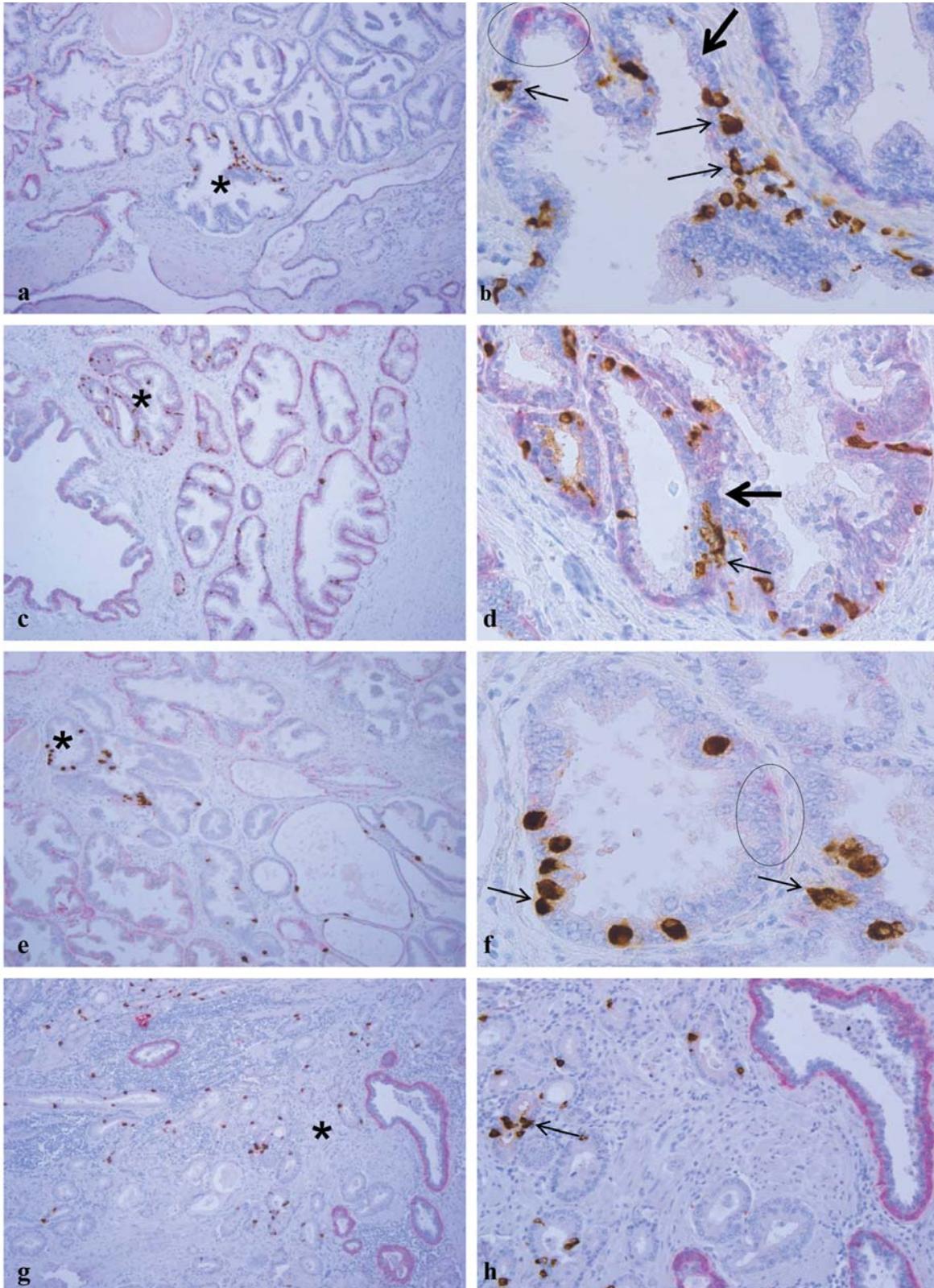


Figure 2. Clusters of CgA-positive cells in selected cases. Paraffin-embedded human prostate tissue sections were double immunostained for CK β E12 (red) and CgA (brown). Thin arrows identify CgA-positive basal or luminal cells. Thick arrows identify focally disrupted basal cell layers. Circles identify residual basal cells. a, c, e, and g: $\times 100$; b, d, f, and h: higher magnification ($\times 400$) of areas marked with an asterisk in a, c, e, and g, respectively.

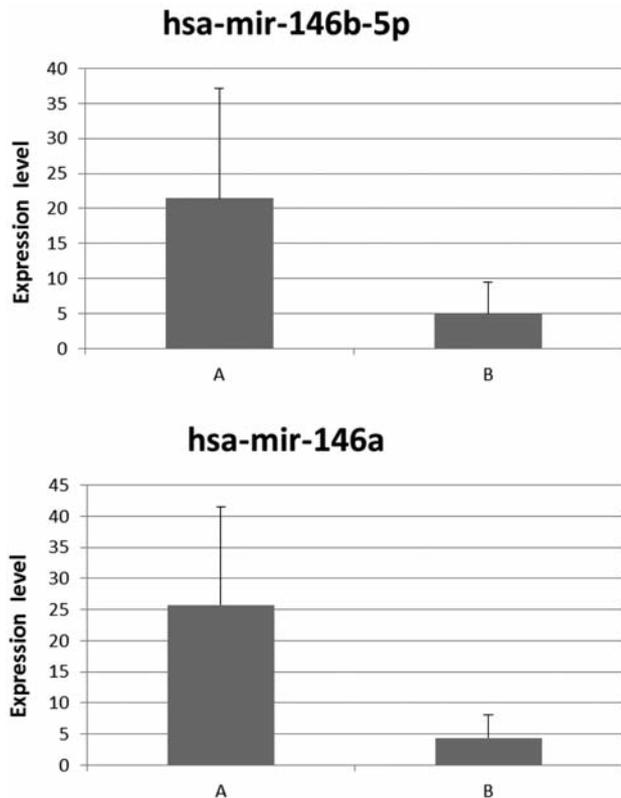


Figure 3. Expression of Has-miR-146a and Has-miR-146b-5p in CgA-negative (A) and positive (B) structures.

neoplasia (PIN), and to invasive or metastatic stage (33-39). Progression from one stage to another is believed to be triggered by increasing accumulation of genetic abnormalities and expression of stage-specific molecules (33-39). These theories are consistent with results of studies in tissue cultures and animal models, whereas are hard to reconcile with the following facts: (i) previous studies revealed that some healthy men between 19- and 29-years-old had a spectrum of prostate lesions, including PIN and incipient adenocarcinoma (40-42), and (ii) recent studies have detected a DNA phenotype in some 'healthy' men and in normal prostate tissues adjacent to prostate tumor, which is identical to that of invasive prostate cancer (43-46). In addition, despite tremendous efforts, the specific mechanism(s) and molecules that trigger progression from one stage to another have not been defined. As yet, although hundreds of tumor progression- and invasion-related markers have been reported, only prostate-specific antigen (PSA) has been approved by the FDA as a clinical diagnostic marker for prostate tumors (47, 48). In addition, although PIN is universally considered as the precursor of invasive and metastatic prostate cancer, it represents a group of heterogeneous lesions with variable clinical presentations and genetic and biochemical profiles

(49-54). A long-term follow-up (11-years) of 789 patients with PIN in the transition/central zone revealed that 36 patients (4.6%) developed prostate cancer during the follow-up (49). A short-term follow-up (2 years) of 207 patients with PIN reported that the incidence of the subsequent development of prostate cancer was 30% for low-grade PIN and 27% for high- grade PIN (50).

The identification of these specific individuals at greater risk for invasive prostate lesions, however, is very difficult, as a vast majority of PIN exhibits no symptoms or aberrant expression of PSA (51-54). In addition, none of the other clinical tests, including Computed tomography scan, ultrasound-guided prostate biopsy, and gene expression profiling, has significant value in predicting which of the PIN lesions may progress. A recent study of p53, c-erbB2, Ki-67, estrogen receptor (ER), progesterone (PR), B-cell lymphoma 2 (BCL2), and markers for angiogenesis in human breast tumors revealed that all markers correlated well with histological grade, whereas none of them was clearly associated with progression from an in situ to invasive stage (55). Thus, the only available method to monitor PIN progression is repeat biopsy, which is costly and painful. Since over 90% of prostate cancer related deaths result from invasion-related illnesses and the incidence of PIN may be as high as 16.5% to 25% in routine or ultrasound-guided prostate biopsy (51-54), there is an urgent need to uncover the intrinsic mechanism of prostate tumor invasion, and to identify the specific tumors or individuals at greatest risk of invasive lesions. It has been well documented that early detection and interventions could significantly improve prognosis and reduce treatment-related costs.

Recent studies have revealed that tumor cell budding (defined as single cells or small clusters of up to four cells within the stromal tissue at the invasive front) reproducibly predicts prognosis and survival of patients with esophageal and colorectal cancer (13-15). A more recent study (56) of 201 patients with adenocarcinoma of the lung further revealed that tumor budding was observed in 78 (43.1%) out of the 181 cases with invasive adenocarcinoma. The presence of tumor budding was significantly associated with lymph node metastasis ($p=0.005$), vascular invasion ($p=0.003$), lymphatic invasion ($p=0.009$), and pleural invasion ($p=0.029$). The overall 5-year survival rates of patients with and without tumor cell budding were 67.5% and 88.3%, respectively ($p=0.0057$). Compared with cancer cells forming nests, budding cells displayed significantly reduced expression of cellular adhesion molecules, E-cadherin and β -catenin ($p<0.05$), but increased expression of laminin 5-gamma2 ($p<0.05$). The morphological and immunohistochemical profiles of CgA-positive cells seen in our current study are very similar to those described in esophageal, colorectal, lung, and breast cancer (13-15,24,56), suggesting that these cells may undergo the same clinical course and have the same consequences.

It is well known that IL-2 expression in activated and anergic T-lymphocytes and the notch signaling pathway regulated by miR-146a and miR-146b-5p are frequently deregulated in many types of cancer, including prostate cancer (57-60). More plausible evidence has been reported recently that miR-146a may function as a tumor suppressor gene in modulating hyaluronan/ the RhoA-kinase 1 (HA/ROCK1)-mediated tumorigenicity in androgen-dependent prostate cancer (57-60). In addition, a genetic variation in pre-miR-146a affects the expression level of mature miR-146a, contributing to the genetic predisposition to prostate cancer (57-60). A number of recent studies have further implicated the possible link between aberrant expression of miR-146a and miR-146b-5p and development of hormone-refractory prostate cancer and migration and invasion of glioma (61-63). Together, these findings suggest that elucidation of the physical integrity of the tumor capsule, and identification of the growth pattern and biological profile of cells overlying focally disrupted tumor capsule may significantly facilitate identification of the specific pre-invasive tumor types or individuals at increased risk of invasive lesions.

The detailed mechanism for the formation of CgA-positive cell clusters preferentially in epithelial structures with focally disrupted basal cell layers is unknown, but is very likely to result from the bio-physiological consequences of such disruptions. As the prostate epithelium is normally devoid of both blood vessels and lymphatic ducts, and the basal cell layer is the sole source of several tumor suppressors, a focal disruption in a given basal cell layer could lead to several focal alterations, including: (i) localized loss of tumor suppressors and paracrine inhibitory functions, which confer epithelial cell growth advantages allowing escape from programmed cell death (64-68); (ii) localized increase of permeability for oxygen, nutrients, and growth factors, which selectively favors the proliferation of progenitor or stem cells (69-71); (iii) localized increase of infiltration of leukocytes, which directly export growth factors to the epithelial cells through direct physical contact (72-77); (iv) direct epithelial stromal cell contact, which augments the expression of stromal matrix metalloproteinase (MMP) or represses the normal production and distribution of E-cadherin and other cell adhesion molecules, facilitating epithelial to mesenchymal transition and cell motility (78-80); (v) direct exposure of the epithelial cells to different cytokines, which facilitates vasculogenic mimicry and tumor angiogenesis (81, 82), and (vi) direct physical contact between newly formed cell clusters and stromal cells, which stimulates the production of tenascin and other invasion-associated molecules that facilitate stromal tissue remodeling and angiogenesis, providing a favorable microenvironment for epithelial cell migration and proliferation (83).

These alterations individually or collectively lead to increasing proliferation and motility in cells overlying such disruptions. More importantly, these alterations selectively favor the proliferation of progenitor or stem cells overlying focally disrupted basal cell layers, or activate mitogen-activated protein kinases and protein kinase C that trigger the exit of the residual primitive stem cells from quiescence. Thus, pre-invasive prostate tumor cells with CgA expression may represent a population of tumor progenitors or a biologically more aggressive cell clone with a greater propensity to progress to invasive tumor.

No definitive conclusions, however, can be drawn from the current study, as our sample size was small, and clinical follow-up data were not available. On the other hand, as the morphological and immunohistochemical profiles of CgA-positive cells seen in our current study are almost identical to those described in esophageal, colorectal, lung, and breast cancer (13-15, 52, 53), it is possible that these cells may undergo the same clinical course and have the same consequences. More importantly, the altered miRNAs detected in our current study not only correlate well with our immunohistochemical and morphological data, but are also consistent with those of recent reports from other studies (58-60). Thus, if confirmed by clinical follow-up or other approaches, our study may substantially facilitate the early identification of patients with specific types of pre-invasive prostate lesions at greater risk of invasion.

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