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14. ABSTRACT Humanin is a recently discovered potent survival peptide encoded by the 16S mitochondrial RNA. We cloned humanin as an insulin-like growth factor binding protein-3 (IGFBP-3) antagonist, and others identified it as a Bax antagonist. We hypothesize that humanin is an important prostate cancer regulator and that it may have a role as a diagnostic or prognostic marker in this disease. We proposed to characterize the role of humanin as a regulator of cell survival, growth, and signalling in prostate cancer in vitro, to define its interactions with IGF related molecules including IGFBP-3. We also proposed to identify alterations in humanin levels in sera and tissues of men with prostate cancer using a serum bank and a CaP tissue array. Our data so far indicates that humanin is present in prostate and seminal plasma, and is increased in prostate cancer specimens and its presence is a poor prognostic factor for disease free survival. As findings indicate that humanin levels are related to outcome in CaP, using humanin assays as a potential prognostic tool in patients with prostate cancer could improve treatment decisions in the future.					
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Introduction

Prostate cancer is the most commonly diagnosed tumor, and the second leading cause of cancer-related deaths in American men. Advanced androgen-independent prostate cancer has essentially no treatment and new therapeutics are urgently needed. Additionally, PSA is currently the only widely available diagnostic marker and its prognostic abilities are very limited making new disease markers essential in this malignancy. Humanin is a recently discovered potent survival peptide derived not from the nuclear DNA as most proteins, but from the mitochondria genome. We have shown that humanin binds and blocks the actions of the apoptotic molecule insulin-like growth factor binding protein-3 (IGFBP-3), and others identified it as an antagonist of another cell death molecule, Bax. Still others have identified humanin as a wide spectrum survival factor for various cell types. It is well recognized that the growth of cancers is determined among other things by a balance between factors that induce cell survival and those that induce cell death. Several growth and survival factors (including members of the IGF, EGF and FGF families) have been identified to date and have led to the development of new cancer treatments. Our preliminary data indicates that humanin is present in prostate, and is increased in prostate cancer specimens. We found that humanin stimulates growth and survival of prostate cancer cells, promotes the actions of androgens that are critical for prostate cancer progression, and enhances prostate tumor survival in mice. We synthesized humanin antagonist peptides that interfere with humanin action and block prostate cancer survival. We hypothesized that humanin is an important prostate cancer regulator and that it may have a role as a diagnostic or prognostic marker in this disease. We further hypothesized that humanin antagonists can interfere with prostate cancer progression and can serve as novel therapeutics in the treatment of advanced prostatic malignancy. We proposed to further characterize the role of humanin in prostate cancer. We also aimed to identify alterations in humanin levels in the sera and tissues of men with prostate cancer. These studies are potentially of great importance as prostate cancer is the leading non-cutaneous malignancy in men; the second leading cause of cancer death in males; and typically progresses from hormone-dependent to an androgen-independent state, thus making hormonal treatment (androgen ablation) ineffective, making new therapeutic and diagnostic approaches essential. The discovery that humanin is an endogenous prostatic growth factor makes it an attractive target for therapy. Additionally, if our findings indicate that humanin levels are related to outcome in prostate cancer, using humanin assays as a potential prognostic tool in patients with prostate cancer could improve treatment decisions in the future. Thus, these expected findings may improve our understanding of this emerging prostate cancer therapy and facilitate further clinical development in men with prostate cancer.

Body

Statement of Work Task 1. To characterize the survival modulating roles of humanin and humanin antagonists in prostate cancer cell models in vitro and to identify their interactions with IGFBP-3.

We have synthesized various humanin mutants and began performing MTT cell growth assays and Caspase-3 and ELISA apoptosis assays on the prostate cell lines 22RV1, LAPC-4 and LNCaP using these various HN mutants and IGFBP-3

Statement of Work Task 2. To identify alterations in humanin levels in sera and tissues of men with CaP.

We completed a staining for humanin in prostate specimens and optimized conditions for immunohistochemistry

We performed IHC staining on prostate cancer tissue arrays. This is discussed in detail below and figures are included.

We perform humanin ELISA assays on a number of biological fluids and improved assay performance.

Statement of Work Task 3. To determine the effects of humanin antagonists on the growth of prostate cancer xenografts in vivo.

We have initiated pilot studies of infusing humanin in mice to determine pharmacokinetics.

Summary of key achievements for Task 2:

Humanin (HN) is a mitochondrial and nuclear-encoded 24 amino acid polypeptide originally discovered from the occipital lobe of Alzheimer's Disease patients (AD) (Hashimoto 2001) where it is found predominantly in the neurons and glial cells (Tajima 2002). Humanin is secreted into the extracellular medium and acts at cell surface binding site triggering a signal transduction cascade. It provides anti-apoptotic protection against cell death by preventing the translocation of Bax from the cytosol to the mitochondria suppressing cytochrome c release (Guo 2003). Recently, we reported that this peptide promoted the survival of Leydig cells in culture by suppression of both Bax and Bid (Colon 2006). As cell survival through anti-apoptotic factors is one of the important characteristics for cancer cell viability, we decided to investigate Humanin's potential meaning in human prostate cancer by examining its in situ expression across a wide spectrum of primary tumors by tissue microarray analysis.

Western immunoblotting was performed on frozen tissues from 10 cases of morphologically normal human prostate tissue and 10 samples of prostate cancer. Immunohistochemistry was performed on tissue microarrays constructed from paraffin embedded primary prostate cancer specimens from 226 hormone naïve patients who underwent radical retropubic prostatectomy. All cases provided informative epithelium and 197 of those included Humanin-informative spots from cancer tissues. In total, 979 tissue microarray spots were informative, including morphologically normal prostate (NL; n=257), benign prostatic hyperplasia (BPH; n=107), prostatic intraepithelial neoplasia (PIN; n=41) and invasive prostate cancer (Cancer; n=574). Both cytoplasmic and nuclear Humanin expression was scored in a semi-quantitative fashion using an integrated intensity measure (0.0-3.0) and positivity (0-100%), respectively. The protein expression distribution was examined across the spectrum of epithelial tissues and its association with standard clinicopathological covariates and tumor recurrence was examined in 184 outcome and marker-informative patients.

Immunoblots revealed a single 3.5 kD band overall stronger from prostate cancer than from normal prostate ($p < 0.001$). Humanin expression is low overall in prostate tissues. 97% of spots had negative to weak cytoplasmic staining (< 1.0), and 92% of spots had infrequent nuclear staining ($< 25\%$). Nonetheless, the mean cytoplasmic Humanin expression was significantly higher in cancer (intensity = 0.11) compared to normal (intensity = 0.045; $p = 0.0028$), and the mean nuclear Humanin expression was also significantly higher in cancer (10.48% positive) compared to normal tissue (2.24% positive; $p < 0.0001$). Nuclear Humanin expression was a significant and negative prognosticator in low grade (Gleason's Score 2-6) prostate cancers when used as either a continuous variable (Cox Proportional Hazards $p = 0.0060$), or dichotomized variable cut at 15% positive ($p = 0.0098$). However, Humanin was not an independent predictor of tumor recurrence in multivariate analysis in this patient substrata including seminal vesicle and capsular involvement, and preoperative PSA as covariates.

We thus concluded that Humanin is expressed at higher levels in prostate cancers compared to matched normal tissues. High nuclear Humanin expression is strongly associated with an increased risk of tumor recurrence. This is the first report examining Humanin in prostate cancer and our findings suggest that it may be a prognostic marker and therapeutic target

Figure 1. Over-expression of HN in human prostate cancer by immunoblot. Western blot analysis of HN in whole prostate tissue protein extracts (100 μg) derived from 10 cases of normal prostate and 10 matched cases of prostate cancer. Representative immunoblots showing bands near 3.5 kD are shown (A.), including a 10 ng positive control lane of recombinant human HN protein. Beta actin was used as an internal loading control for normalization (not shown). Bar graphs represent three independent experiments (B.). The relative HN expression from prostate cancer (CaP) and morphologically normal prostate (NI Pros) is significantly different ($p < 0.001$ by Students T test) by densitometric quantification (OD: optical density, represents the mean \pm SD). Also shown in B is humanin staining in a mouse model of prostate cancer also indicating that humanin is overexpressed in CaP.

A

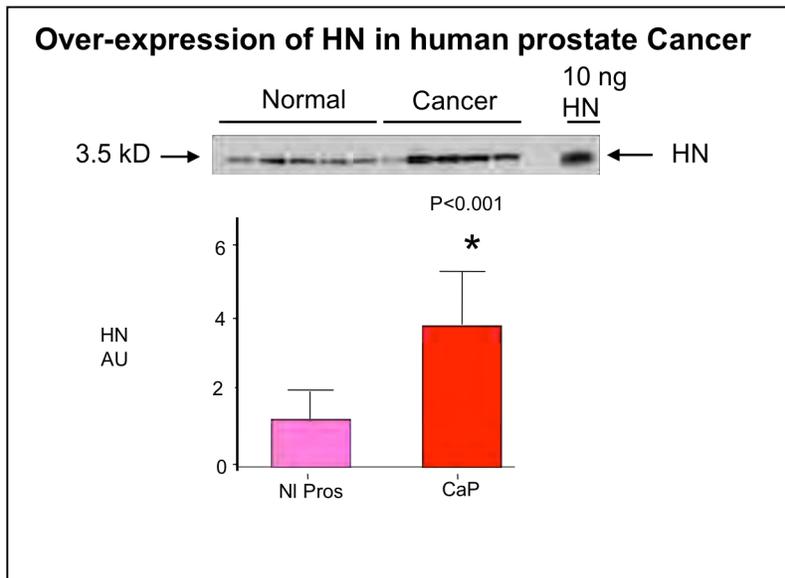


Figure 1B

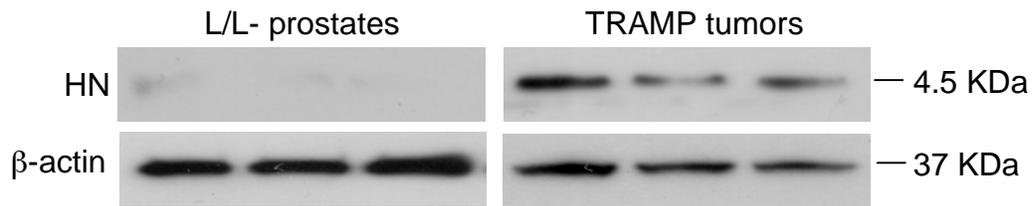


Figure 2. Immunohistochemical localization of Humanin protein in human testis. Application of anti-Humanin antibody by immunohistochemistry demonstrates cytoplasmic staining of Leydig cells (A, B), as well as occasional Sertoli cells and spermatids of the seminiferous tubules (D, E). Replacing primary anti-Humanin antibody with non-immune pooled rabbit IgG at an equivalent concentration serves as negative control (C, F), note the absence of staining. (A, B 100X; B, C, E, F 400 X magnification).

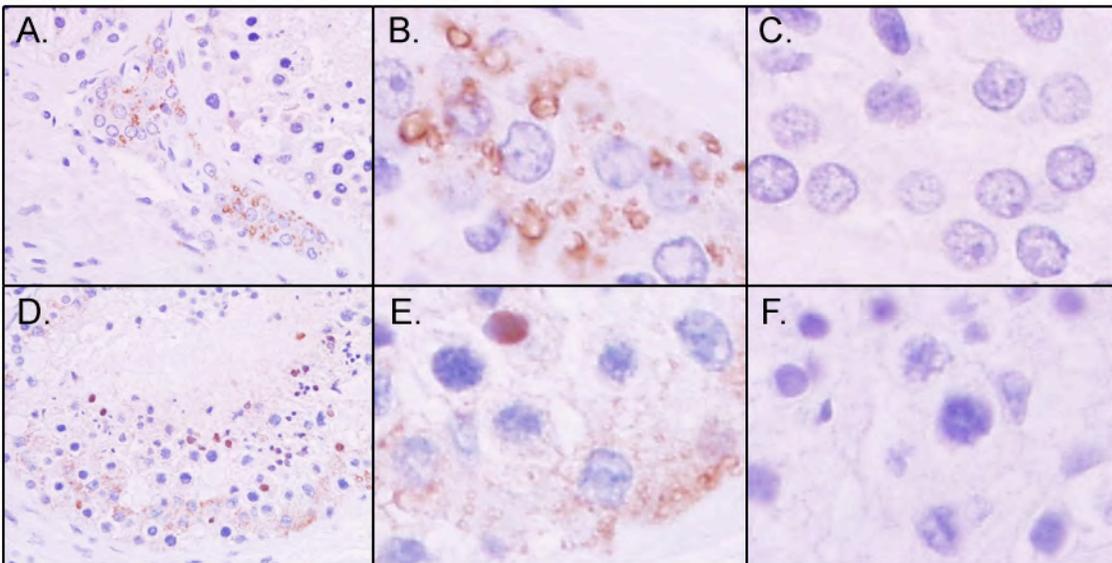


Figure 3. Immunohistochemical staining patterns of Humanin protein on human prostate tissue microarrays.

Various patterns of immunohistochemical staining for Humanin protein is seen on representative prostate tissue samples. Normal tissue showing weak cytoplasmic epithelial staining of glandular cells (A.). Staining in basal cells is frequently higher than that seen in glandular cells. Negative staining of prostate adenocarcinoma (B.). An example of staining of both nuclei and cytoplasm in cancer (C.). Staining of nuclei alone with an increasing positivity (D.-F.). Cytoplasmic staining without nuclear expression, shown with increasing intensity (G.-I.). Regional and focal cytoplasmic staining (J., K.). Coarsely granular cytoplasmic staining seen here in normal prostate (L.), and malignant epithelium (M.). Stromal fibromuscular cells lacking significant expression (B., D., E., G., J.), showing moderate staining (A., C., H., M., N.), and strong staining (I., O.). (100X magnification with 400X inserts).

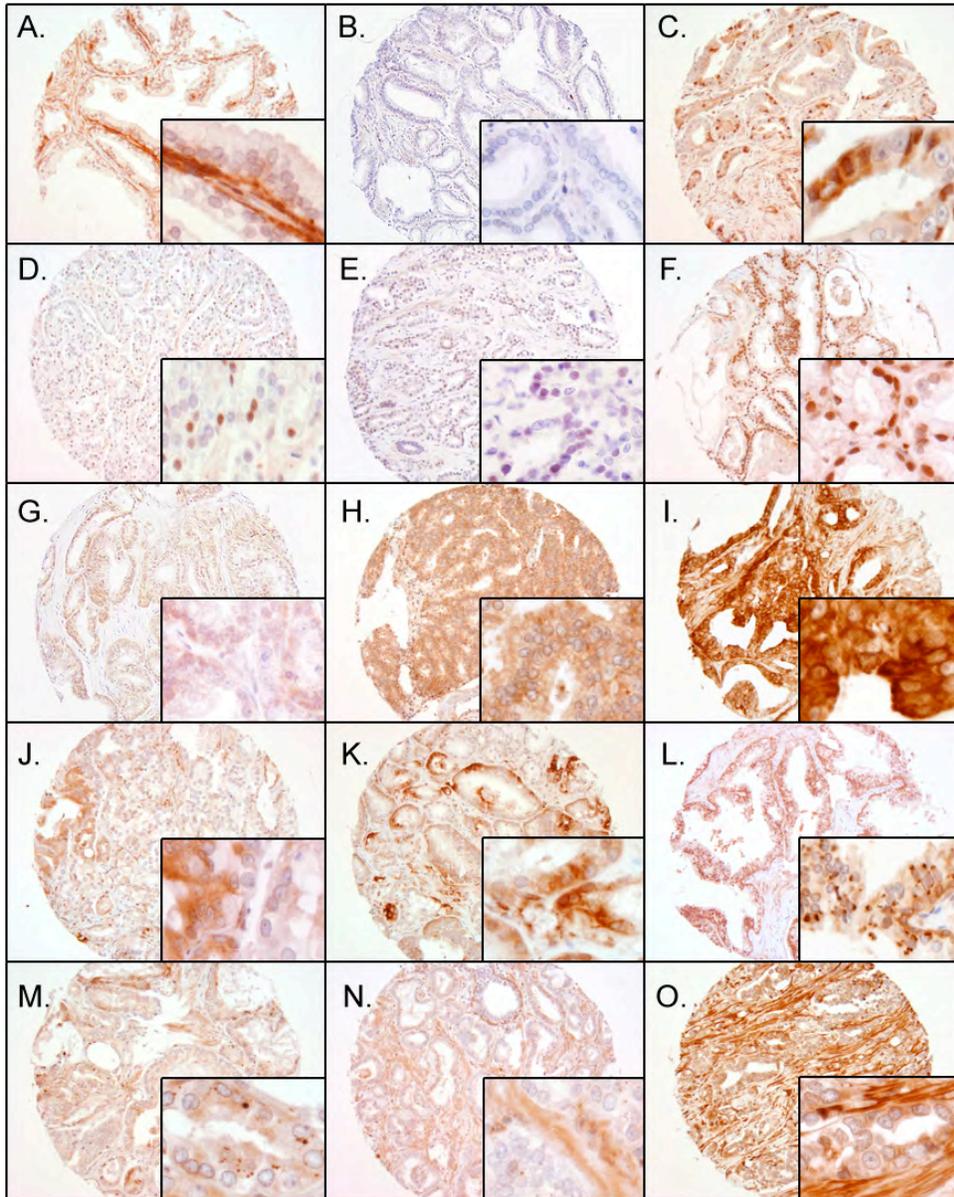


Figure 4. Humanin protein expression distribution on the prostate tissue microarray stratified by histological category and Gleason's grade. The distribution of Humanin protein measured as cytoplasmic intensity (0-3) and nuclear positivity (0-100%) by immunohistochemistry as seen in 979 informative primary tissue microarray spots is shown in A. and D., respectively. 84% and 67% of all samples were negative for cytoplasmic and nuclear staining, respectively. Mean Humanin cytoplasmic and nuclear expression stratified by histologic category is seen in B. and E., respectively. Error bars represent 1 standard error. The mean cytoplasmic expression was significantly higher in cancer (ProsCa) compared to normal (NL; $p=0.028$), and benign prostatic hyperplasia (BPH; $p=0.0017$). Humanin expression in prostatic intraepithelial neoplasia (PIN) was significantly higher than BPH ($p=0.028$). The mean nuclear Humanin expression was significantly higher in cancer compared to PIN ($p=0.0003$), normal ($p<0.0001$), and BPH ($p<0.0001$). Humanin expression in NL was significantly higher than BPH ($p=0.039$).

The cytoplasmic and nuclear expression in 574 primary prostate cancer tissue spots is shown as boxplots in C and F, respectively. The mean cytoplasmic Humanin intensity for Gleason's grades 1-2 ($n=91$), 3 ($n=326$), 4 ($n=130$) and 5 ($n=27$) were, 0.030, 0.11, 0.18 and 0.011, respectively. Grades 3 and 4 were significantly higher than grade 2 ($p=0.010$ and $p=0.0032$, respectively). The mean nuclear Humanin positivity for grades 1-2, 3, 4 and 5 were, 8.90, 11.35, 8.92 and 12.78, respectively. There were no significant differences in nuclear Humanin expression across the spectrum of Gleason grades by unpaired t-test (all $p>0.05$).

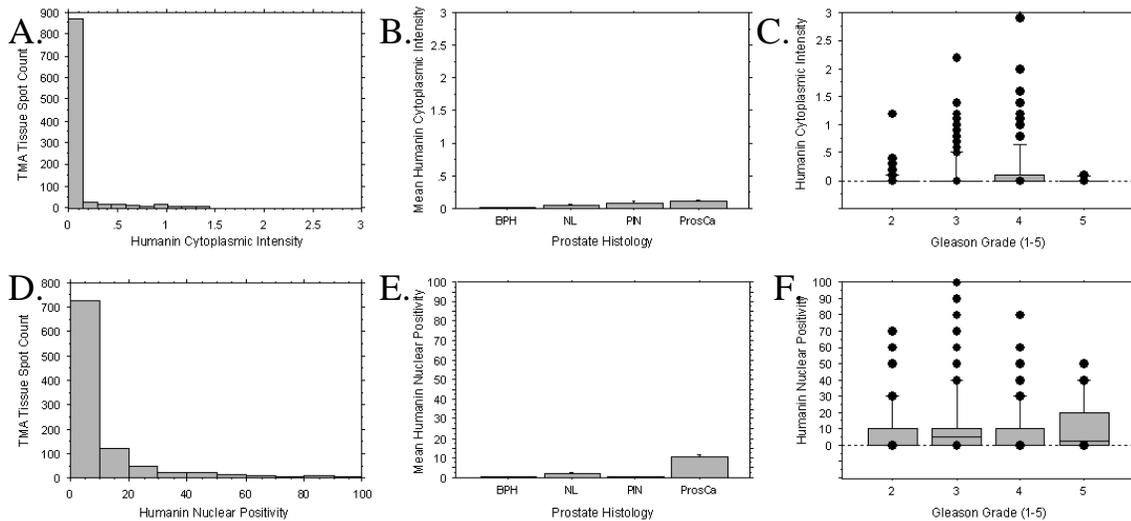
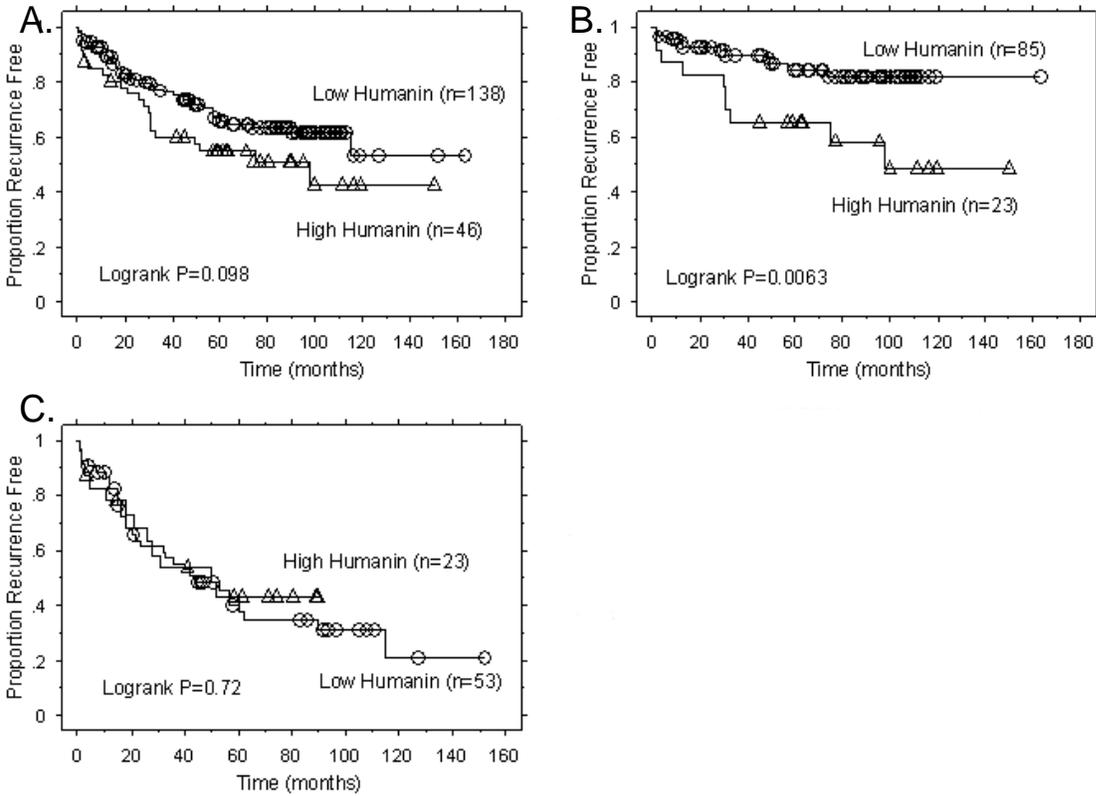


Figure 5. Kaplan-Meier curves for time to prostate cancer recurrence. Kaplan-Meier Curves for time to tumor recurrence stratified by nuclear Humanin protein expression status (n=184 patients) are seen in all patients (A.), and in patients stratified by either low tumor grade (B.) or high tumor grade (C.). Gleason's Scores of 2-6 and 7-10 are considered low and high grade, respectively. Humanin nuclear positivities of ≥ 15 and < 15 are considered "High" and "Low" Humanin, respectively. A high nuclear expression phenotype in low grade cancers is associated with a higher risk of developing recurrent prostate cancer ($p=0.0063$). Censored times marked by circles and triangles.



Key Research Accomplishments

- Establishment of humanin immunohistochemistry in prostate cancer specimens as a quantitative assay that can be applied to Pathology clinical practice.
- Establishment of humanin as an over-expressed protein in human prostate cancer specimens
- Demonstration of the anti apoptosis molecule humanin as being nuclear in its location as a poor prognostic indicator in prostate cancer
- Refinement of the humanin serum assay

Reportable Outcomes

- Liu B, Cohen P. Humanin is involved in prostate cancer growth and survival, in vitro and in vivo. Abstract Presented at the Annual Meeting of the Endocrine Society Meeting 2006.
- Colon E, Strand ML, Carlsson-Skwirut C, Wahlgren A, Svechnikov KV, Cohen P, Soder O. Anti-apoptotic factor humanin is expressed in the testis and prevents cell-death in leydig cells during the first wave of spermatogenesis. *J Cell Physiol.* 2006; 208:373-85

Conclusions

Thus, we conclude that humanin is a new potent survival factor with potential implications in prostate cancer. In our findings here we presented evidence that it is elevated in prostate cancer tissues and that it acts to promote CaP survival *in vivo*. The increased staining of nuclear humanin in the specimens of prostate cancer patients obtained at surgery and analyzed with up to >10 years follow up using a tissue array of prostate cancer indicates that humanin is involved in the process of disease progression and that it is a potential clinically useful marker for aggressive disease. Together with additional ongoing experiments we have proceeded with establishing a role for humanin in the diagnosis and treatment of prostate cancer.

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- Liu B, Cohen P. Humanin is involved in prostate cancer growth and survival, in vitro and in vivo. Abstract Presented at the Annual Meeting of the Endocrine Society Meeting 2006.
- Colon E, Strand ML, Carlsson-Skwirut C, Wahlgren A, Svechnikov KV, Cohen P, Soder O. Anti-apoptotic factor humanin is expressed in the testis and prevents cell-death in leydig cells during the first wave of spermatogenesis. *J Cell Physiol.* 2006; 208:373-85
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