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Differential Expression of Zinc Transporters in Prostate Epithelia of Racial Groups

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In the award (W81XWH-08-1-0627) the stated goals are to study the zinc transporters that may be disparately expressed in the prostate tissues of African American (AA) males as compared to European American (EA) males. This may lead to identification of the potential molecular targets for preventive or therapeutic measures or nutritional, environmental or life style factors for their potential relationship to the incidence or progression of prostate cancer in various racial groups. The following tasks are to be carried out in a synergistic fashion between the laboratories of Dr. Bagasra (Claiifin University) and Dr. Balla (University of Illinois, Chicago). The prostate tissues, pre-made tissue microarrays (TMAs) and primary cell lines will be obtained from the NCI -initiated “Cooperative Prostate Cancer Tissue Resource (CPCTR)” by Dr. Balla, one of the P.I.s of the present proposal and of the CPCTR. The main tissue microarray being used is composed of age-matched and the Gleason score-matched prostate tissues with representative tissues from 150 African Americans and 150 European American racial groups. Other TMAs are also used to address questions of whether hZIP gene and protein expression is associated with poor outcome and/or Gleason grade. Significant progress has been made in each of the three TASKS proposed. So far we have two peer-reviewed publications as well as numerous oral and poster presentations have resulted. We also have three additional publications in preparation for peer-review journals.

Prostate Cancer, zinc, zinc transporters, African-American, European-American, microRNA, health disparity
# Table of Contents

- Introduction ........................................................................................................... 4
- Body ......................................................................................................................... 5
- Key Research Accomplishments ........................................................................... 15
- Reportable Outcomes ............................................................................................ 15
- Conclusions .............................................................................................................
- References ............................................................................................................... 18
- Appendices .............................................................................................................. 19
Second Year Progress Report
Differential Expression of Zinc Transporters in Prostate Epithelia of Racial Groups

Introduction: In the award (W81XWH-08-1-0627) our goals are to identify an explanation for differences in prostate cancer incidence and outcome in African Americans (AAs) at the molecular levels. The main question that we are attempting to answer is “Are there any consistent differences in the expression of significant genes or proteins in the prostate cancers taken from AAs versus those from European Americans (EAs)?” Because there is a well-documented depression in zinc levels with neoplastic conversion of normal prostate cells, the study had initially focused on the expression of genes known as zinc transporters. The hypothesis is that the expression levels of one or more of zinc transporter genes (hZIPs), as measured by mRNA expression may be differentially expressed in AAs and EAs. A study of the genes and proteins which influence the expression of any gene confirmed to be disparately expressed might lead to the identification of one or more environmental or living pattern factors worthy of epidemiological research for its potential relationship to the incidence or progression of prostate cancer in AAs, EAs or other ethnic groups.

For this purpose we study the zinc transporters that may be differentially expressed in the prostate glandular tissues of AA males as compared to EA males. This might lead to identification of the potential molecular targets for nutritional, environmental or life style factors.

The following tasks are carried out in a synergistic fashion between the laboratories of Dr. Bagasra (Claiiln University) and Dr. Balla (University of Illinois, Chicago). The prostate tissues, pre-made tissue microarrays (TMAs) were obtained from the NCI-initiated “Cooperative Prostate Cancer Tissue Resource (CPCTR)” by Dr. Balla, one of the P.I.s of the present work and of the CPCTR. The main tissue microarray that we are using is composed of age-matched and the Gleason score-matched prostate tissues with representative tissues from 150 African Americans and 150 European American racial groups. Another type of TMA, based on long term clinical follow up was used to address questions of whether hZIP gene and protein expression is associated with poor outcome and/or Gleason grade.

Task 1: To measure the differential expression of four main zinc transporters- hZIP1, hZIP2, hZIP3 and hZIP4 –genes by RT-in situ PCR as well as by real time PCR in the tissue arrays (TMA) prepared from various racial groups with age-matched, Gleason Score-matched in order to determine any potential differences in the gene expression at the mRNA between the racial groups and normal vs neoplastic clusters of cells.

Task 2: To determine the differential levels of the amount and location of intracellular zinc in various areas of the prostate gland as well in cells in different histological grades of cancer and in healthy prostate tissues by utilizing fluorescent zinc indicators and other methods.

Task 3: To develop antibodies against the four zinc transporters, carry out an extensive quality control protocol for these antibodies. We will perform immunohistochemical analyses of TMAs and determine the degree of each of the four hZIP proteins expression by semi-quantitative image analyses. Note: Task 3 was eliminated from the grant by recommendation of the study section before grant was awarded.
**Body:**

**TASKS 1 & 2: TASK 2:** We have already completed a series of optimizations experiments to carry out this work. We have initially utilized prostate cancer cell lines and now we are optimizing the experimental conditions on frozen tissue sections. This work is in progress and we have completed the first series of work during the third fiscal year of the grant and we are now measuring the differential expression of four main zinc transporters- hZIP1, hZIP2, hZIP3 and hZIP4 –genes by RT-in situ PCR as well as by real time PCR in the tissue arrays (TMA) prepared from various racial groups with age-matched, Gleason Score-matched in order to determine any potential differences in the gene expression at the mRNA between the racial groups and normal vs neoplastic clusters of cells. An important part of the Task #1 is to carry out the RT-in situ PCR for hZIPs and determine if: a) there is a differential expression of hZIPs in stromal vs. glandular tissues and b) to determine the differential expressions of hZIPs in various racial groups by real time PCR for the four zinc transporters (hZIP1, hZIP2, hZIP3 and hZIP4).

**AIM #1a:** In order to carry out this aims we first had to go through extensive quality control to be certain that our RT-in situ PCR is able to differentiate the relative down or up-regulations of hZIP1 gene. We recently published an article in a Peer-Reviewed Journal “Methods”. A summary of the studies is presented below and the PDF of the article is attached.

**ABSTRACT:** Zinc (Zn) is essential for a very large number and variety of cellular functions but is also potentially toxic. Zn homeostasis is therefore dynamically maintained by a variety of transporters and other proteins distributed in distinct cellular and subcellular compartments. Zn transport is mediated by two major protein families: the Zip family, which mediates Zn influx, and the ZnTs which are primarily linked to Zn sequestration into intracellular compartments and are, thereby, involved in lowering cytoplasmic Zn free ion concentrations. In the prostate epithelial cell, the accumulation of high cellular zinc is a specialized function that is necessary for these cells to carry out the major physiological functions of production and secretion of prostatic fluids. The loss of Zn accumulation is the most consistent and persistent characteristic of prostate malignancy. Currently, there are no direct methods to determine the relative Zn levels in various cell types of prostate gland (i.e. stroma, glandular epithelia, acini, muscular, etc) and no reliable ways to compare the Zn in normal versus malignant areas of the gland. Here we report a new method to show a differential Zn staining method that correlates with various stages of prostate cancer development in situ and expression of a human Zn transporter1-hZIP1 -in situ by in situ reverse transcriptase-polymerase chain reaction hybridization (ISRTTPCR) that correlate with the relative Zn levels determined by the differential Zn staining method. By utilizing these methods we show for the first time that: 1) the relative Zn levels are very low to absent in the malignant glands, 2) normal glands show high Zn levels in both glandular epithelia as well as in stromal tissues, 3) the Zn levels begin to decrease in pre-malignant glands and precedes the development of malignancy, 4) the expression of human Zn transporter1 (hZIP1) appears to correlate with the Zn levels in the prostate glands and may be the major Zn regulator in this organ.

Fresh frozen sections from 42 male prostate biopsies with a clinical history of prostate cancer, and 6 from autopsy specimens with normal glands from patients who died from automobile accidents were processed for RT-in situ-PCR [16-17]. Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. In this study, **Figure 1A** shows the high level of cellular Zn that characterizes the normal glandular epithelial cells (green color in **Fig 1A**). In contrast, the stroma exhibits relatively lower levels of zinc. Therefore, the in situ Zn staining utilizing two different color indicators with different affinity and intracellular threshold provides the differential Zn accumulation between normal glandular epithelium and stroma [18-19]. The marked reduction of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are apparent in **Figure 1 B and C**. Similar pattern is also seen in patient with adenocarcinoma Gleason score 3+3 (moderately differentiated) in **Figure 1D**. Like the expression of hZIP1, the loss of Zn occurs early in malignancy. Due to the depletion of Zn in the malignant glands, the stromal Zn level gives the appearance of
relatively higher Zn levels. Many studies have observed that Zn levels are greatly decreased in extracts of resected malignant tissue preparations [20]. However, our present study provides the first in situ detection of the depleted cellular Zn levels in adenocarcinomatous glands as compared to the high Zn levels in normal glandular epithelium. Of note, the decrease in Zn level in the malignant glands is due to a decrease in the cellular accumulation of zinc. This suggests that the decrease in intracellular zinc, and not impaired secretion of Zn into the lumen (prostatic fluid), is principally responsible for the decrease in malignant tissue Zn level. Thus, the results of our study are consistent with previous studies [6-10, 21].

Correspondingly, Figure 2 the relative expression of mRNA expression for hZIP1 were determined in the 42 prostate resections. The typical results represented in Figure 2 were consistently observed in the frozen sections of all 42 prostate resections. The results show that hZIP1 gene expression is evident uniformly in the epithelium of the normal peripheral zone glands and is relatively low in the stroma (Fig 2A). hZIP1 expression is markedly down regulated to the extent of not being demonstrable in the two high grade adenocarcinomatous glands (red colors of the glandular epithelia in Fig 2B-C) and in moderately differentiated adenocarcinoma glands (Fig 2D); however, it is present in the stromal tissues but at much lower levels as compared to the normal control (Fig 2A).

Figure 3 shows the hZIP1 expression profiles in the frozen sections of the normal glands adjacent to malignant glands in two of the patients (Fig 3A and B). As one can observe in Figures 3A and Figure 3B, on the right side of each slide there are mostly normal appearing glands that exhibit relatively strong yellow/green staining for hZIP1 expression and as one moves toward the left the degree of expression of hZIP1 decreases as the tumor grade of adenocarcinomatous glands begin to increase. In the same patient (Figure 3B) as one moves further to the left (Figure 3C), one can easily recognize lower grade tumor and relatively higher degree of hZIP1 expression. In this section, one can also note the overall increase in the relative hZIP1 expression.

Figure 1: Zinc Levels in Prostate Tissue Frozen Sections. Representative Zinc Levels in Prostate Sections, Figure 1: Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. High Zn is represented by Newport Green, yellow/green stain and low Zn is represented by TSQ red stain. A) Figure 1A shows normal prostate gland from a 42 year old subject. Of note, the high level of cellular Zn indicated by dark green staining with new port green Zn indicator dye that characterizes the normal glandular epithelial cells (black arrows, green color in Fig 1A). In contrast, the stroma exhibits relatively lower levels of zinc, indicated by less intense green color in the stroma (white arrows). Therefore, the in situ Zn staining utilizing two different color indicators with different affinity and intracellular threshold provides the differential Zn accumulation between normal glandular epithelium and stroma [18-19]. The marked reduction of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are shown in Figure 1B and C. The malignant region of the peripheral zone shows a significant depletion of Zn in the malignant glandular epithelium as exhibited by the red staining (white arrows) in three patient’s resected tissues. Here, one notes relative depletion of Zn indicated by TSQ red Zn indicator dye and relatively higher levels of Zn in the stromal areas. Similar relative depletion of Zn is also observed in Figure 1D pattern is also seen in patient with
adenocarcinoma Gleason score 3+3 (moderately differentiated) in Figure 1D. H & E sections from the same specimens are shown on right side of the slide. (Final magnification 100x)

Figure 2 (Figure A, B, C): In situ detection of hZIP1 mRNA expression levels in Frozen Prostate Sections from one normal (Fig 1A) and three prostate cancer subjects (Fig B-D) are shown. Figure 2A shows relatively high expression of hZIP1 in a normal prostate section, both in the glandular as well as in the stromal areas. This figure shows the relative degree of expression of hZIP1 as determined by in situ RTPCR/Hybridization method. As one notes that in all three specimens from malignant tissues (B, C and D, white arrows), the malignant areas of the prostate, represented by abnormal glandular epithelia, exhibits a significant down regulation of hZIP1 mRNA as compared to the surrounding stromal areas showing a relatively higher degree of hZIP1 expression (black arrows). H & E sections from the same specimens are shown on right side of the slide. (final magnification100x).
Figure 3: In situ detection of hZIP1 mRNA and the frozen sections of normal and malignant glands in the same tissue sections: Figure 3A and Figure 3B
Analyses of hZIP1 expression by in situ RTPCR/hybridization are shown from two patients. Relatively high levels of hZIP1 expressions in normal appearing glands can be seen as a yellowish/green color on the right portions (white arrows) of the slides whereas low or absent expression can be seen as red colors in malignant glands on the left sides of the slides (black arrows). Of note, the greenish color in the stoma in Figure 3A and Figure 3B are absent, suggesting low expression of hZIP1. In the same patient (Figure 3B) as one further moves to the left (Figure 3C) towards relatively normal appearing area, one can easily recognize higher expression of hZIP1 (shown in greenish color and more normal appearing glands (black arrows). H & E sections from the same specimens are shown on right side of the slide.

Brief Comments and Further work to be completed: Worldwide, there are more than 10 million new cancer cases each year, and cancer is the cause of approximately 12% of all deaths. Among all cancers, PC is the second leading cause of male cancer related deaths [22-23]. Over 200,000 males were identified with PC in 2003 and as a result ~30,000 died. In 2010 more than 186,000 US men will be diagnosed with PC and over 30,000 may die. Despite the extensive clinical and experimental studies over the recent decades, the pathogenesis of PC remains unanswered [reviewed in 1-2]. The interaction of genetics and the environment and its influence on the molecular mechanisms responsible in the development and progression of malignant prostate cells are largely unknown [24-26]. There is a great need to explore the role of differential gene expression that leads to altered cellular metabolism as an essential factor in prostate malignancy [27-27]. The combination of genetic/molecular/environmental factors and their relationships are required to identify the critical events in the prostate malignancy process. Such studies are proving to be very useful in the understanding of the molecular pathogenesis of prostate cancer [6-7, 28].

Zinc and Zn transporters play an important role in the molecular pathogenesis of PC [6-7, 20-21]. PC afflicts one out of nine men over the age of 65 years. Prostatic intraepithelial neoplasia (PINs) is relatively common and occurs early in life [1-2]. However, progression to invasive carcinoma is significantly less common. What are the factors that cause PIN to become invasive? It appears that race and ethnicity is also an important factor! PC disproportionately affects AA men, who, along with black Jamaican men, have the
highest PC incidence rates in the world [22-23, 29-34]. In addition, AA men develop PC significantly earlier and at the time of diagnosis they are present with the higher-grade adenocarcinoma than the age-matched EA men [31-35].

At the global level, rates of incidence are low in Asian and African men, low-to-moderate in EA men, and highest in AA men [22-23, 30, 32]. Using data collected between 1988 and 1992, Wingo, et al. [36] reported that AAs have a 35% higher incidence rate and a 223% higher mortality rate from PC as compared with EAs. Similar data has been shown by others [22-23]. The differences in incidence and mortality between AAs and EAs have been attributed to both environmental and biological factors [6-7, 27-29]. When compared with EA men, AA men present at a younger age, with higher grade (Gleason Score), and stage of disease at the onset of age, and with a greater delay in diagnosis [37-39]. Whether the pathogenesis of PC is different in AA men as compared to EA men remains unanswered. Whittemore, et al., [40] have noted that AA men appear to have a larger volume of “latent” PC load. These investigators believe that larger-volume latent carcinomas are those that progress to become clinically evident at a faster rate, suggesting that events that account for racial differences in PC incidence may occur very early in cell transformation and thus may be genetically controlled [33, 35]

**Role of Zinc in the Pathogenesis of PC:** The normal human prostate gland has an unusual capability of accumulating high levels of zinc; generally about ten-fold higher than other soft tissues. This capability resides within the mitochondrial organelles of glandular secretory epithelial cells of the peripheral zone (PZ). PZ is the main region where PC first appears. Conversely, the central and transitional zones contain relatively very low levels of zinc, except in benign prostatic hyperplasia [BPH, reviewed in 6-7, 8 10]. Over five decades of clinical studies have consistently demonstrated that prostate cancer tissue samples consistently contain about 65% less Zn than normal prostate tissue. More precisely, the Zn concentration (nmols/gram wet weight) of a normal peripheral zone tissue approximates 3,000–4,500; malignant peripheral zone tissue approximates one-tenth of that level (400–800); and other soft tissues approximate 200–400. Consequently, malignant prostate tissue Zn levels are decreased by ~70–85% compared to normal peripheral zone, and the decrease is observed in the glandular epithelial cells. Most importantly, one rarely, if ever, finds malignant glands that have retained the high Zn levels that characterize the normal gland. In addition, the decrease in Zn occurs early in the development of prostate malignancy [6-7]. These established clinical relationships have raised important issues that relate to the role and mechanisms of Zn accumulation in the normal functioning of the prostate gland and the loss of Zn accumulation as a requirement in the development of prostate malignancy.

It has been shown by Costello and Franklin groups that the functional role of Zn accumulation is to inhibit citrate oxidation of the highly specialized secretory epithelial cells, which permits the production and secretion of unusually high levels of citrate as a major component of prostatic fluid [7, 10]. In addition, high Zn levels in the mitochondria inhibits terminal oxidation, truncating the Krebs cycle, hence deceeding the ATP-based energy production and resulting in growth/proliferation and inducing mitochondrial apoptogenesis [12, 15]. And this process subsequently inhibits tumor invasion [15]. The combination of such effects can be characterized as anti-tumor effects, which lead us to propose that Zn is a tumor-suppressor agent against prostate cancer. This provides the explanation for the requirement that malignant cells lose the capability to accumulate Zn and the basis for the absence of malignant glands that retain high levels of zinc.

This has led us to pursue the critical issues regarding the mechanism of Zn accumulation in the normal epithelial cells along with the mechanism for the lost ability of the malignant cells to accumulate zinc [7]. The members of the Zip family of Zn transporters have been identified as important Zn transporters for the cellular uptake and accumulation of Zn in mammalian cells. More specifically, we have identified three hZIPs (hZIP1, 2 and 3) that are downregulated [29]. However, hZIP1 has shown to be the most important Zn uptake transporter in prostate cells [6-7, 15].

In our present report, by utilizing two different methods: one that can differentiate the relative low versus high amounts of intracellular Zn by utilizing specific Zn binding molecules *in situ* and another one that can
differentiate the relative degree of \textit{hZIP1 in situ} by ISRTPCR, we demonstrate that Zn is depleted from the neoplastic as well as pre-neoplastic prostatic glandular epithelial cells. Correspondingly, \textit{hZIP1} is expressed in human normal and hyperplastic prostate glandular epithelium; and is down-regulated in adenocarcinomatous glands. Previously, our group has identified the down regulation of \textit{hZIP1} expression in the high prostate cancer at-risk African American male population as compared with European American males in a small number of patients we tested [29]. In this report, for the first time, we show down-regulation of \textit{hZIP1} in a much larger group of patients and also show that Zn accumulation is very low in the adenocarcinomatous glands.

Currently, our laboratory is carrying out the analyses of PC microarray Tissue with other three \textit{hZIPS} (\textit{hzip1}, \textit{hzip2} and \textit{hZIP3}). We are confident that these analyses will be completed by the end of 3\textsuperscript{rd} year.

\textit{Data related to Differential Expression of hZIPS in AAs Versus EAs}

We also evaluated 19-pairs of age and Gleason score matched prostate tissues from AA men \textit{versus} EA men for \textit{hZIP1} expression. The representative photographs are shown in Figure 4. As it is apparent, from Figure 4 that as compared to the prostate glandular tissues of EA men, the \textit{hZIP1} expression in the glandular areas of AA men is markedly downregulated. Since, we are unable to precisely quantify the number of copies of \textit{hZIP1 in situ} by the ISRTPCR method, we cannot state with certainty that in the prostatic glands of AA men \textit{hZIP1} expression is significantly downregulated as compared to age and Gleason score matched EA men, however, utilizing the exactly identical experimental conditions for ISRTPCR for all 19 pairs it is remarkable that in all 19 pairs we observed the similar finding.
Correspondingly, Figure 5 shows the matched pairs with differential Zn accumulations in the prostate resections of AA men versus EA men. Again, we can easily observe the differences in the relative accumulations of Zn in the two racial groups. The relative low amount of Zn observed in the prostatic epithelia of AA men is striking as compared to the matched corresponding tissues of the EA men.

Currently, we have performed extensive microdissections of frozen tissues normal as well as malignant tissues from the prostate resection of AAs versus EAs to separate out glandular and stromal tissues. We have isolated mRNAs from these specimens and we are now comparing the relative expressions of hZIP1-4 by real time PCR.

In addition, we recently published a paper in the journal Prostate (PDF attached) where we and our collaborators at the Armed Forces Institute of Pathology (AFIP) measured zinc concentrations in the prostate of subjects with and without recurrence of disease after prostatectomy. Zinc levels were lower in the normal appearing prostate tissue of patients with bad outcome: Zinc measurement by ICP-MS in a nested case-control study of 80 paraffin-embedded prostate tissues showed prognostic significance, as patients with PSA recurrence had lower zinc. See Graph 1 below.
Graph 1: Zinc concentrations were lower in the normal appearing prostate tissue of patients with bad outcome (p= 0.04, n=80 Wilcoxon signed rank paired test). Note that there are more dots (pairs of subjects) that fall on the right side of the dividing line than on the left side.

**TASK 3: Immunohistochemical expression of hZIP1 protein expression.**

ABSTRACT Dr. Balla’s laboratory has acquired 6 commercially available monoclonal antibodies (mAbs) to hZIP1 and is carrying out extensive immunostaining in order to evaluate these antibodies. The Sigma Co. (St. Louis, MO) monoclonal antibody showed better results staining of epithelial cells and some staining of stromal cells with minimal background. The other antibodies are from Alpha Diagnostics and a series of monoclonal antibodies from Santa Cruz Laboratory (one commercially available and three not yet released to the general market). Two optimization TMAs were also built during year 2 of the project in Dr. Balla’s lab and were used for extensive analyses. Based on best staining results with the Sigma Co. antibody we used it to perform the following experiments using the CPCTR Outcomes-based TMA:

hZIP1 immunohistochemical (IHC) expression was not a prognostic marker and did not correlate with prostatic zinc concentration: The TMA study of 147 pairs of tumors (294 patients) showed no difference in hZIP1 expression in this nested-case control cohort of recurrence vs. non-recurrence (p = 0.123, Wilcoxon paired test). In 64 patients where we measured tissue zinc concentration by ICP-MS and hZIP1 IHC expression, there was no correlation between zinc levels and hZIP1 expression r_s= 0.-0.046; p = 0.36).

Even though this TMA is not specifically built for the study of racial disparities, it does have a substantial number of African American subjects. Therefore we used the African American subset of subjects and their Caucasian matched controls to address hZIP1 IHC expression. We compared matched 36 pairs of African Americans and European Americans. There was no significant difference by the Wilcoxon signed rank paired test (p= 0.07; a larger cohort with 150 pairs is planned after antibody specificity re-evaluation). Due to this unexpected result, we have since then (during the 3rd year and current year of the grant) performed further characterization of this antibody for specificity using Western blots. To our surprise, none of the 6 commercially available antibodies reacts with hZIP1 in cancer cell lines that have hZIP1 expression by RT-PCR (data not shown, work performed in Dr. Larisa Nonn’s laboratory in our Department). Since then, Dr. Nonn has obtained rabbit antibodies from Drs. Franklin and Costello laboratories, and discovered (last month) that their antibody indeed reacts with hZIP1 by Western blot, confirming the original claim of Franklin and Costello’s
laboratory. We are now in the process of staining the Outcomes-based CPCTR TMA and the Ethnicity-based CPCTR with this antibody and should have new data in the next couple of months.

**New Finding in the area of microRNA:**
Mentioned in the original grant proposal, not as a specific task, but as something that would need investigation, is the issue of what could be the reason (mechanism) to explain racial differences in the expression of zinc transporters. Since there are no known highly prevalent mutations of this gene, it was hypothesized that the differences could be due to micro RNA regulation of hZIP1 mRNA.

Using five different online sites, we identified putative miRNAs that target the 3’UTR of hZIP1 with bioinformatics. We than compared those putative miRNAs with published data sets showing miRNA changes that occur in PCa tissue. From this screen, we selected six miRNAs to test; miR-96, miR-223, miR-346, miR-30c, miR-100 and miR-182. To examine the role of these miRNAs in hZIP1 regulation, we compared the level of each miRNA to hZIP1 mRNA in laser-capture-microdissected (LCM) normal and tumor prostate tissue. Tissue was collected from 10 patients, five Caucasian-American and five African-American. MiRNA and mRNA levels were measured by qRT-PCR with Taqman™ assays. A positive result would show inverse correlation between the miRNA and its target mRNA; i.e. the mRNA levels is down in PCa and the miRNA level is up in PCa. Our results showed that only miR-182 showed inverse correlation with hZIP1 mRNA in the tissue and the correlation was only present in the Caucasian tissue (Spearman rho=−0.77, p=0.009) (Figure 6). In the PCa tissue from Caucasian patients the Spearman correlation was perfect at -1.0, p=0.003. Although the inverse correlation is strongly suggestive of regulation of hZIP1 by miR-182, further in vitro validation was required.

![Figure 6](image)

**Figure 6.** MiR-182 levels have inverse correlation with hZIP1 mRNA in Caucasian patients (Spearman rho = -0.77, p = 0.009)

By qRT-PCR, miR-182 and hZIP1 levels were measured in cell cultures of normal prostate and PCa cell lines. hZIP1 mRNA and miR-182 were only present in the epithelium-derived cells, both normal and PCa, but not in the prostate stromal cells. hZIP levels were lower in the PC3 PCa cell line, which was derived from androgen-independent metastasis, whereas hZIP1 levels were equivalent in two normal epithelial and the LNCaP cell line. LNCaP was also derived from a metastasis, but it is dependent upon androgen for growth, albeit with a mutant form of the androgen receptor. An inverse miR-182 expression pattern was seen in the cell cultures (Figure 7). To directly test the ability of miR-182 to inhibit hZIP1 mRNA, pre-miR-182 was transfected into the cells. Following transfection, miR-182 levels increased and hZIP1 mRNA levels decreased.
This data strongly implicates miR-182 and possibly the other microRNAs (miR-183 and miR-96) that express as a cluster with miR-182 as key regulators of hZIP1 levels.

Figure 7. Inverse correlation between micro RNA-182 and hZIP1 mRNA expression in ten different primary and established cancer cell lines ($r = 0.77$, $p = 0.009$).

The data thus far suggests regulation of hZIP1 by miR-182 only in the Caucasian-Americans. The mechanisms for this race-specific finding are not clear at this point in time, but remain a priority for future studies. Ongoing studies are aimed and validating the hZIP1-3’UTR binding site for miR-182 via mutagenesis in a luciferase vector and functional assays on zinc uptake in miR-182 transfected cells. In addition, we plan to examine miR-182 levels among races and hundreds of PCa specimens by tissue microarray and in situ hybridization and we be applying for funds for this project shortly.

Figure 8: hZIP1 mRNA expression after transfection with miRNA-182 and other microRNAs that are expressed as a cluster with miRNA-182.

A manuscript on the micro RNA study is ready now and it should be sent to JBC before the end of May, 2011.

The Proposed Timetable of the Proposed Research: All three Specific Aims were initiated immediately after the initiation of the funded proposal. Since all three aims are interdependent, we carry out all three aims simultaneously in two different laboratories (at CU and UIC). All of the samples have already been obtained during the current, third year. The main function of UIC in this synergistic effort is to provide tissues and pathology expertise. Experiments utilizing sectioned material (zinc content and TMA zinc transporter expression levels) have been performed and extensive optimization and antibody validation procedures have been carried out. The project is on track.
Additional Insights:
1) The reason why hZIP1 has less expression in African Americans is not known. Apparently, it is not a mutation of the hZIP1 gene; therefore we have to look for other mechanisms. We hypothesized in the original grant proposal that it could be caused by differential methylation or differential microRNA function. Since the beginning of the award period we have addressed the latter possibility. With the help of Larisa Nonn, PhD, an expert in microRNAs at UIC Department of Pathology, we have studied six microRNAs that could be the regulators of hZIP1 expression. We find differences already, but they need to be compared by hZIP1 gene expression (at CU) and protein expression (at the UIC).

2) We realized that isolating total mRNAs from the prostate tissues from AAS and EAs and determining the relative expression levels of two ethnic groups may not reveal anything important since the degrees of expressions of hZIP1 differ significantly from area to area and specimens to specimens and between glandular vs stromal areas. Therefore, we decided to carry out extensive microdissections of the tissues. In order to carry out this new task we have purchased an Eppendorf Microdissection System at Claflin University. We are also using a Leica laser microdissection dissection instrument at UIC for this purpose.

Key Research Accomplishments:
- The relative degree of hZIP1 is differentially expressed in the prostate tissues.
- The hZIP1 is downregulated in the glandular malignant tissues of the prostate
- The hZIP1 is upregulated in the stromal areas in the malignant glands.
- It appears that the degree of upregulation of hZIP1 is higher in AAs stromal areas as compared to EAs
- It also appears that the downregulation of hZIP1 is relatively higher in the glandular areas of AAs as compared to EAs.
- The microdissection of glandular and stromal tissues and isolation of total mRNAs from 30 normal prostate glands (16 from AAS and 14 from EAs) is being carried out at CU and may given us the definite answer regarding the race and ethnicity differences of hZIP1 and other hZIPs.
- The microRNAs that regulate hZIP1 and possibly other zinc transporters have been identified.
- We now established that low zinc concentrations in prostate tissues next to malignancy is lower in patients with biochemical recurrence.

Reportable Outcomes:

The Research Associate, Leslie A. Johnson (who was graduate student last year has carried out extensive has carried out bulk of the studies and we have been able to publish our key findings in a Peer-reviewed Journal (Methods). Dr. Andre Balla, the partnering PI has already provided tissues and TMA for the first two tasks; and we have been able to complete the key elements of our research

Training of Minority Students: During the last two years the PI at CU hired three minority graduate students. All these graduate students were recent graduates of MS in biotechnology. Miss Johnson has been working with Dr. Bagasra during her graduate education and now is working as Research Associate and carrying out the bulk of the experiments. Mr. Kendall Williams worked on this project during the last year and now moved on to Chemistry Department working on Biodefence project also funded by DoD. Miss Meaghen Ashby graduate this year and started Medical School at the Medical University of South Carolina in August 2010.

1) During the 2008-2010 academic-years Dr. Bagasra the following undergraduate and graduate students in prostate cancer and other related research (breast cancer, diabetes and miRNA research connected to
prostate cancer). Each of the undergraduate student presented their research at various conferences as detailed below and all the graduate students have publications in peer-reviewed journals;

**Cindy Lewis:** Miss Lewis interned last year at the Cleveland Clinic and now has been awarded a Full Graduate Scholarship for her doctoral work

**Leslie A. Johnson:** Miss Johnson was Dr. Bagasra’s graduate student and she was hired as an Associate Research Scientist to work on the DoD funded project. She has won numerous awards for her presentations and has carried out significant part of the proposed project. She plans to continue her doctoral degree next year and work on Health Disparity issues.

**Kendall Williams:** Mr. Williams joined Dr. Bagasra’s lab to assist him on the DoD project. After one year he joined Department of Chemistry to work on a Biodefense project, also funded by DoD.

**Keaira Berry:** Miss Berry has been Dr. Bagasra’s undergraduate student since her freshman year and won numerous awards for her presentations on prostate cancer. She has joined our graduate program in Biotechnology.

**Sharne Morrow:** Miss Marrow graduated from Claflin with BS in biology. She worked on prostate cancer project. Currently, she is working and preparing for her GRE to enter graduate school.

**Kanack MA:** Mr. Kanak was a graduate student until May 2010 and worked closely with Dr. Bagasra. He was hired as Research Associate in Dr. Bagasra’s lab and now working on a Biofuel Project funded by DARPA.

**Alseiari MA, MD:** Dr. Alseiari was a post doctoral fellow in Dr. Bagasra’s lab for one year and assisted on HIV vaccine project. Currently, he is an internal medicine resident at the MGH, in Boston, MA

**Addanki KC:** Mr. Addanki is a graduate of our first batch of graduate students and has received a specialization in Forensic DNA analyses. He will be responsible for DNA analyses in our newly constructed Forensic DNA lab.

**Mayank Aggarwal:** Mr. Aggarwal is a 2008 graduate of Claflin University’s MS in Biotech and currently perusing his Ph.D. in Biomedical Science at University of Florida.

**Azima Kalsum:** Miss Kalsum was Dr. Bagasra’s graduate student and she was hired as an Associate Research Scientist to work on a army- DoD funded project on Biodefence and currently working in Department of Chemistry.

**Bianca Thomas:** Ms Thomas is a senior undergraduate student, currently working with Dr. Bagasra on Prostate cancer as well as on diabetes and role of zinc transporters.

**Jazzmine Clemons:** Miss Clements is a junior undergraduate student, currently working with Dr. Bagasra on Prostate and breast cancers and role of zinc transporters.

**Jessica Abercrombie:** Miss Abercrombie is a junior undergraduate student, currently working with Dr. Bagasra on Prostate and breast cancers and role of zinc transporters.

**Clara L. Jones:** Miss Jones is a sr. graduate student currently, working with Dr. Bagasra on Prostate and breast cancers and role of zinc transporters.

**Sian Ramlal:** Miss Ramlal is a sr. undergraduate student currently working with Dr. Bagasra on Prostate and breast cancers and role of zinc transporters.

**Publications from CU:** The following publications have resulted from the current DoD CDMRP funded award:

5. Bagasra. O. “Insight into the Inhibitory Effect of HHV-6, HHV-7 and GBV-C co-infection: Role of Homologous miRNAs in Downregulations of Viral Replications” JIDC Annual Conference. Alghero, Sardinia, It. May 17th 08.
7. Bagasra O, Aggarwal M, Addanki K. “Inhibitory Effect of HHV-6, HHV-7 and GBV-C co-infection: Role of Homologous miRNAs in Downregulations of Viral Replications”. 33rd Feb Meeting in Athens, Greece, Abst # PPSA-1, June 28-July 3rd, 08.


10. Johnson Leslie (2009). Molecular Pathogenesis of Prostate Cancer in Relation to the African American Community, the Role of Zinc and Zinc Transporters: Environment and Genetic Influences. 2nd Annual James E. Clyburn Health Disparities Lecture: Social Determinants of Health: Framing the Issues at the University of South Carolina in Columbia, SC.


12. Kanack MA, MS (BioTech); Alseiari MA, MD; Addanki KC, MS; Aggarwal M, MS (BioTech); Noorali S, PhD; Kalsum A, MS (BioTech); MPH; Mahalingam K, PhD; Pace D.G. PhD and Bagasra O, Triplex Forming microRNAs Form Stable Complexes with HIV-1 provirus and Inhibit Its Replication. 2010 Anti-viral Applications of RNAI. Madrid, Spain, May 4-8. 2010-Antiviral Applications of RNA Interference.


19. Bianca N. Thomas, Leslie A. Johnson, Jacob Sterling, and Dr. Omar Bagasra. The Effects of Zinc Accumulation pertaining to Diabetes in the African American community vs. other racial populations Claflin University, Orangeburg, SC March 27, 20102nd Annual Open House & Research Day (*won Second Prize in Poster Competition).


21. Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Kendall Williams, Joseph P. Pestner, & Dr. Omar Bagasra Role of Zinc Transporters and the Role of Zinc in Breast Epithelia of various racial groups. 3rd Annual James E. Clyburn Lecture Series University of South Carolina, Columbia April 9, 2010.

22. Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Kendall Williams, Joseph P. Pestner, & Dr. Omar Bagasra Role of Zinc Transporters and the Role of Zinc in Breast Epithelia of various racial groups Nobel Laureate Chemistry (Dr. Martin Chalfie) Lecture Series Claflin University, Orangeburg, SC April 20, 2010.

23. Keaira Berry, Leslie A. Johnson, Kendall Williams, Andrea’ Kajdacsy-Balla, Omar Bagasra Differential expression of Human Zinc Transporters 1 (hZIP1) in nonmalignant prostate tissue: Racial Differences Nobel Laureate Chemistry (Dr. Martin Chalfie) Lecture Series Claflin University, Orangeburg, SC April 20, 2010.


26. Leslie A. Johnson, Keaira Berry Kendall Williams, Andrea’ Kajdacsy-Balla, Omar Bagasra Prostate Cancer: A health disparity among African American men-The racial differences within nonmalignant prostate tissue pertaining


29. Sian Ramlal, Dr. Samina Noorali and Dr. Omar Bagasra Silencing of HLA-Transcripts expression via RNAi in Jurkat T Cell Lines ABRCM 2010

30. Leslie A. Johnson, Kendall M. Williams, Keaira Berry, Mazhar A. Kanak, Andrea’ Kajdacsy-Balla, Joseph P. Pestaner, and Omar Bagasra’ Prostate Cancer: A health disparity among African American men: Differential expression of hZIP1 within nonmalignant prostate tissue reveals the role of genes and environment. ABRCM 2010


Miss Johnson also won the following awards for her presentations:

**Student Travel Awards, Leslie A. Johnson:**
GlaxoSmithKline Achievement Award Recipient, 2009
Imaging the Pancreatic Beta Cell Travel Award Recipient (NIH), 2009
Prostate Cancer Symposium Travel Award Recipient (NIH), 2009

**Undergraduate Student who won travel awards to attend and present their research findings under DoD training:**


2) Bianca Thomas. The effects of zinc accumulation on diabetes in the African American community versus the European American community. the Annual Biomedical Research for Minority Students (ABRCAMS:2009)

3) Sharme Morrow. The effects of zinc accumulation on Breast Cancer. the Annual Biomedical Research for Minority Students (ABRCAMS: 2009)

**Peer Reviewed Publications:**


**Other Publications not related to this project (2008-2010)**


Publications from UIC: Publications since last annual report DoD CDMRP funded award. Drs. Macias and Balla are funded by this DoD grant award:


Methods 2::316-21, 2010

6. Ananthanarayanan V, Deaton RJ, Amatya A, **Macias V**, Luther E, **Kajdacsy-Balla A**.
   Gann PH Subcellular Localization of p27 and Prostate Cancer Recurrence: Automated Digital Microscopy. *Human Pathology* (Published online ahead of print Feb 1, 2010).


**Undergraduate Student Training at UIC**

1) Jarna Shah: Sophomore at UIC, working on hZIP1 receptor immunohistochemistry optimization and validation in tissue microarrays, Summer 2009 and Fall 2009.
2) Ekaterina Khromatsova: Junior at UIC, working on hZIP1 regulation by putative miRNAs, since beginning of this grant award. Spring 2009 and Fall 2009.

**Resident Training:**

1) Nicoleta Arva, MD, PhD: PGY3 resident in Pathology at UIC, working on hZIP1 regulation by putative micro RNAs and hZIP1 receptor immunohistochemistry optimization and validation in tissue microarrays and full section slides, since beginning of the grant award.

**Travel Awards:**

Dr. Bagasra presented a paper at the AACR: The Science of Cancer Health Disparities. (Abstract # B55. Feb 3-6, 2009) and travel to Carefree, AZ.

None at UIC.

**Conclusions and future plans:** From the above data one can conclude that if our primary hypothesis is correct we need to separate the stromal vs. glandular tissues by microdissections. And, we need to confirm that zinc transporter expressions are actually over-expressed in the stromal tissues of AAs and downregulated in the glandular tissues of AAs as compared to EAs.

**REFERENCES:**


Appendices (two):
