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14. ABSTRACT

In the award (W81XWH-08-1-0626), 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 the identification of the potential molecular targets for preventive or therapeutic measures, 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 (Claflin 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 to be used is composed of age-matched and the Gleason score-matched prostate tissues with representative tissues from 150 African Americans males and 150 European American males. Other TMAs will be 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 in the essence of one peer-reviewed publication as well as numerous oral and poster presentations. We currently have three additional publications in preparation for peer reviewed journals.**

15. SUBJECT TERMS
Prostate Cancer, zinc, zinc transporters, African-American, European-American, microRNA, health disparity

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First Year Progress Report
Differential Expression of Zinc Transporters in Prostate Epithelia of Racial Groups

Introduction: In the award (W81XWH-08-1-0626), our goals are to identify an explanation for differences in prostate cancer incidence and outcome of African American (AA) males at the molecular level. 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 depletion 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 the identification of the potential molecular targets for nutritional, environmental or life style factors.

The following tasks are to be carried out in a synergistic fashion between the laboratories of Dr. Bagasra (Claflin University) and Dr. Balla (University of Illinois, Chicago). The prostate tissues, pre-made tissue microarrays (TMAs) 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 to be used is composed of age-matched and the Gleason score-matched prostate tissues with representative tissues from 150 African American males and 150 European American males. Another type of TMA, based on long term clinical follow up will be 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, we will perform RT-*in situ* PCR as well as real time PCR in the tissue arrays (TMA) prepared from various racial groups which are age--, pTNM stage-, and Gleason Score-matched in order to determine any potential differences in the gene expression at the mRNA level between the racial groups and in 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 as 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, we will 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* protein expressions by semi-quantitative image analyses.

Body: Progress Report

TASKS 1& 2: *We have already completed a series of optimization 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 hope to complete the first series of work within the next 3-6 months. We hope to also in this time frame 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 which are age- Gleason Score-matched in order to determine any potential differences in the gene expression at the mRNA level between the racial groups and in 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 the 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 have just published an article in a Peer-Reviewed Journal "****Methods****". A summary of the studies is presented below and the PDF of the manuscript is attached.*

All references of the summary below are cited in the attached document

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 the 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 the expression of a human Zn transporter1-*hZIP1 -in situ* by *in situ reverse transcriptase-polymerase chain reaction hybridization (ISRTPCR)* 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 in the malignant glands, 2) normal glands show high Zn levels in both glandular epithelia and 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 who died from automobile accidents, were processed for RT-*in situ*-PCR. 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 stromal . 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 the 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. 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.

Correspondingly, in **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 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.

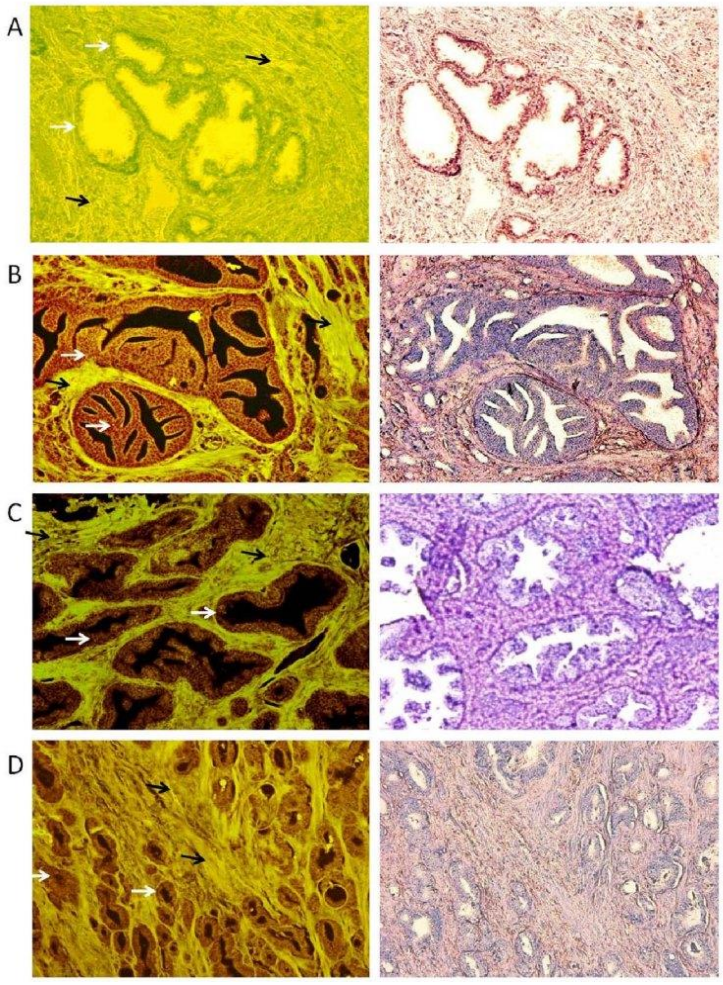


Figure 1: Zinc Levels in Prostate Tissue Frozen Sections. Representative Zinc Levels in Prostate Sections, Figure 1*: Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral 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 1 B 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)*

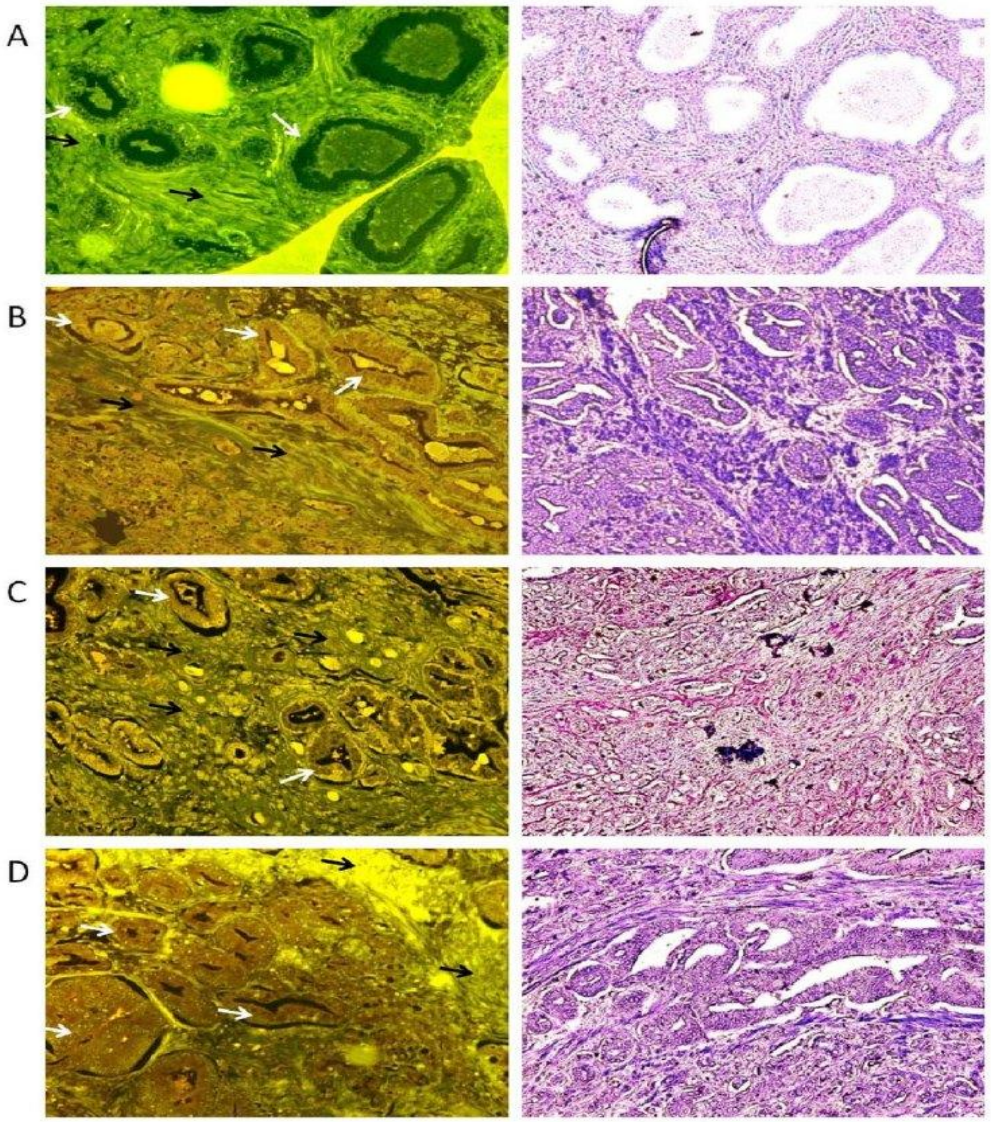


Figure 2 (Figure A, B, C): In situ detection of hZIP1 mRNA expression levels in Frozen Prostate Sections *from one normal (****Fig 1A****) and three prostate cancer subjects (****Fig B-D****) are shown* ***Figure 2A*** *shows relatively high expression of hZIP1 in a normal prostate section, both in the 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 the right side of the slide. (final magnification100x).*

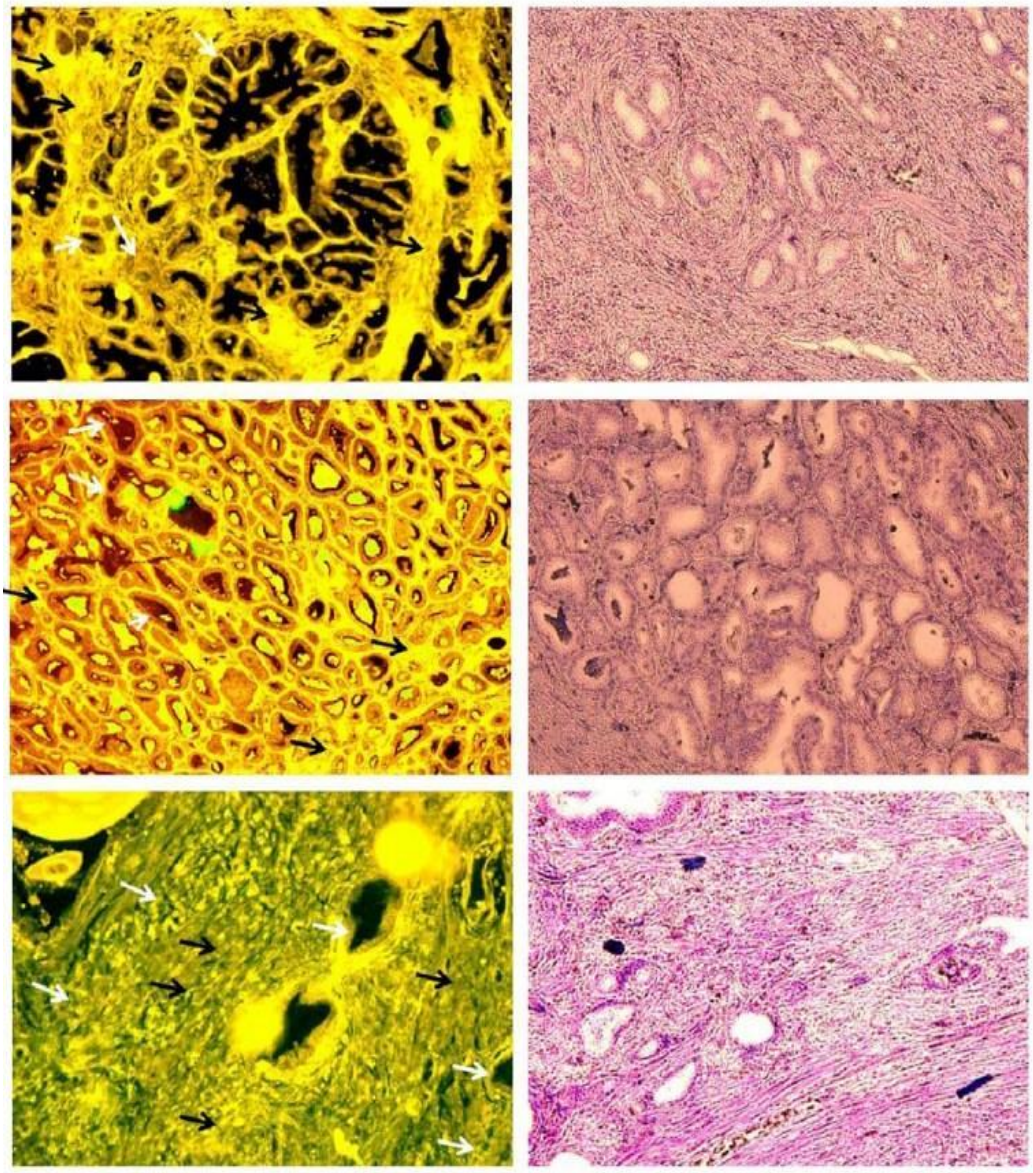


Figure 3: In situ detection of hZIP1 mRNA and the frozen sections of normal and malignant glands in the same tissue sections:
Figure 3A and Figure 3B) *Analyses of hZIP1 expression by in situ RTPCR/hybridization are shown from two patients. Relatively high levels of hZIP1 expressions in normal appearing 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 the 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. 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. 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. 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. 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.

Zinc and Zn transporters play an important role in the molecular pathogenesis of PC. 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. 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. 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.

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. Using data collected between 1988 and 1992, Wingo, *et al.* 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. The differences in incidence and mortality between AAs and EAs have been attributed to both environmental and biological factors. 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. Whether the pathogenesis of PC is different in AA men as compared to EA men remains unanswered. Whittemore, *et al.*, 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.

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. 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. 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.

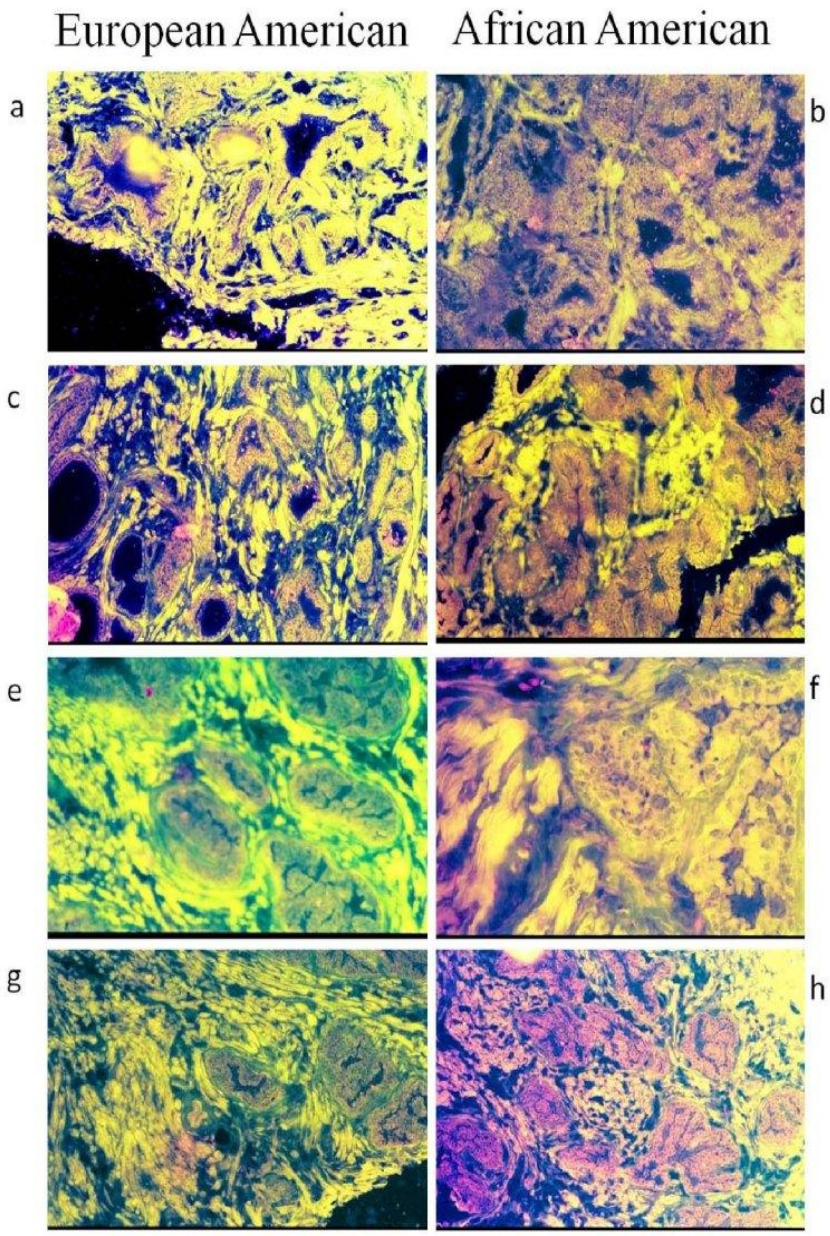
It has been shown by Costello and Franklin group that the functional role of Zn accumulation is to inhibit citrate oxidation of the highly specialized secretory epithelial cells, which permits the production and secretion of unusually high levels of citrate as a major component of prostatic fluid. In addition, high Zn levels in the mitochondria inhibits terminal oxidation, truncating the Krebs cycle, hence decreasing the ATP-based energy production and resulting in less growth/proliferation and inducing mitochondrial apoptogenesis. And this process subsequently inhibits tumor invasion. 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. 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. However, *hZIP1* appears to be the most important Zn uptake transporter in prostate cells.

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 *hZIP1 in situ* by ISRTPCR, we demonstrate that Zn is depleted from the neoplastic as well as pre-neoplastic prostatic glandular epithelial cells. Correspondingly, *hZIP1* is expressed in

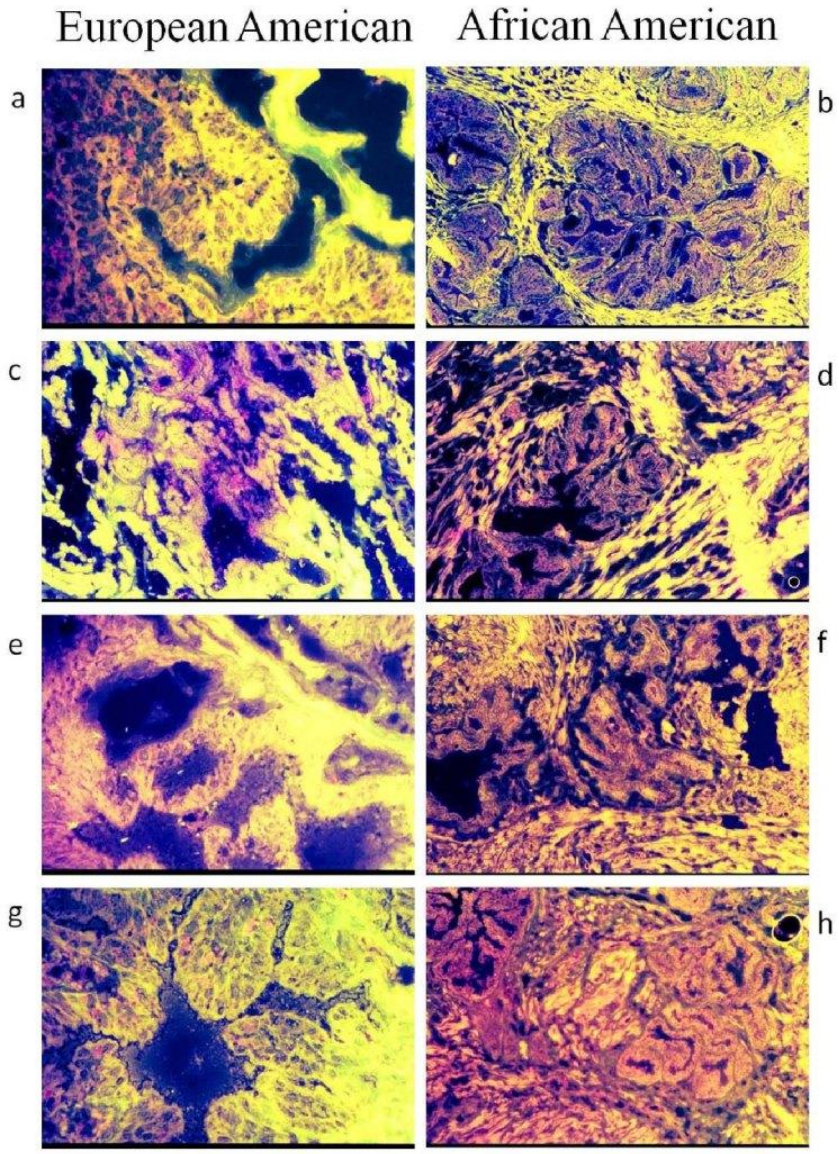
human normal and hyperplastic prostate glandular epithelium; and is down-regulated in adenocarcinomatous glands. Previously, our group has identified the down regulation of *hZIP1* expression in the high prostate cancer at-risk African American male population as compared with European American males in a small number of patients we tested. In this report, for the first time, we show down-regulation of *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 tissue microarray with three other hZIPs (*hZIP1, hZIP2 and hZIP3*). We are confident that these analyses will be completed by the end of 3rd year.

Unpublished Data related to Differential Expression of hZIPs in AAs Versus EAs (Privileged Communication)



We also evaluated 19-pairs of age and Gleason score matched prostate tissues from AA men *versus* EA men for *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 *hZIP1* expression in the glandular areas of AA men is markedly downregulated. Since, we are unable to precisely quantify the number of copies of *hZIP1 in situ* by the ISRTPCR method, we cannot state with certainty that in the prostatic glands of AA men *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.

Figure 4: In situ detection of hZIP1 mRNA and the frozen sections of Gleason Score and age matched malignant glands of European American male (left) and African American male (right). A simple visual analysis indicates a marked down-regulation of the hZIP1in African Americans than European American tissues. However, a more accurate analysis will be required. Microdissections of glandular and stromal tissues are being carried out presently.



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 are carrying out 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 will isolate mRNAs from these specimens and compare the relative expressions of *hZIP1-4* by real time PCR.

Figure 5: In situ detection of zinc by zinc indicator. The frozen sections of Gleason Score and age matched malignant glands of European American (left) and African Americans (right). A simple visual analysis indicates downregulation of the zinc in the African Americans as compared to European American tissues. However, a more accurate analysis will be required. Microdissections of glandular and stromal tissues are being carried out presently.

TASK 3: This part of the project was cut by the DoD from the original budget because at that time there were no commercially available anti-hZIP antibodies. They are currently available today, therefore we decided to proceed with the original plan. Dr. Balla's laboratory has already acquired monoclonal antibodies (mAbs) to hZIP1 and is carrying out extensive immunostaining. This mAb shows good staining of epithelial cells and some staining of stromal cells (**Figure 5)** and is undergoing extensive additional optimization with different antigen retrieval methods that might allow even better staining. The immunohistochemistry procedure is currently undergoing additional validation steps.

The current, not yet validated studies in TMAs, showed that hZIP1 immunohistochemical (IHC) expression was not a prognostic marker and did not correlate with prostatic zinc concentration: A tissue microarray (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 $rs= 0.-0.046$; $p = 0.36$). Because of these unexpected results we are now further characterizing this antibody for specificity. We have also acquired hZIP1 antibody from two other commercial sources and we will also characterize them. It is well known that some monoclonal antibodies do not react against the antigen they are raised against.

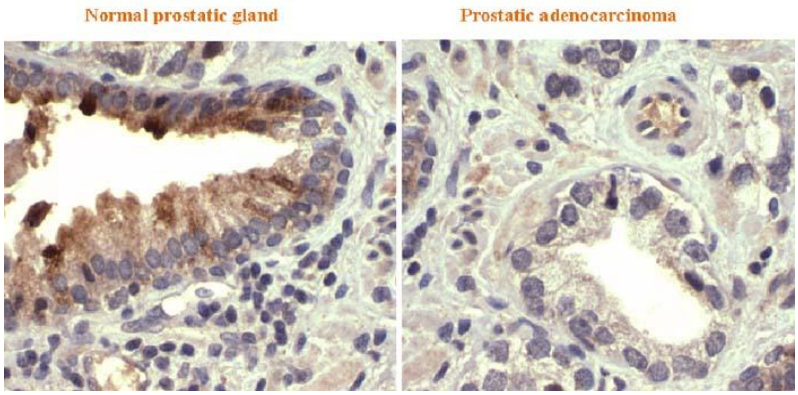


Figure 7. Immunohistochemistry images of hZIP1 expression. Note the decrease in the zinc transporter in cancer gland. The specificity of this and two other new antibodies to hZIP1 are currently being tested

New Finding in the area of microRNA:

We tested the hypothesis that *hZIP1* mRNA and protein levels are decreased in PCa by microRNA(s).

Using five different online sites, we identified putative miRNAs that target the 3'UTR of *hZIP1* with bioinformatics. We then 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 1**). 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.

By qRT-PCR, miR-182 and hZIP1 levels were measure 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 (**Figure 2A-B**). hZIP levels were lower in the PC3 PCa cell line, which was derived from and 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 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 2B**). 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 (**Figure 2C-D**). This data strongly implicates miR-182 as a key regulator of hZIP1 levels.

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, 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.

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). We anticipate that the largest number of the samples will be obtained during the second year. Experiments utilizing sectioned material (zinc content and TMA zinc transporter expression levels) have been performed during the first year and extensive optimization has been carried out.

Additional Insights:

1) The reason for why *hZIP1* has lesser 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 the two ethnic groups may not reveal anything important since the degrees of expressions of *hZIP1* differ significantly from area to area (as shown in **Figures 4 and 5)**, specimen to specimen, 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 a Eppendorf Microdissection System at Claflin University.

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 Gleason score and age-matched EAs' prostate tissues.
- It also appears that the downregulation of *hZIP1* is relatively higher in the glandular areas of AAs as compared to EAs.
- The total isolation of mRNAs from AAs and EAs may be reveal the root cause of differential expression of hZIPs. It will require microdissections of glandular and stromal tissues to figure out the pathogenesis at the molecular levels.
- 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.*

Reportable Outcomes:

The Research Associate, Leslie A. Johnson (who was a graduate student last year has carried the bulk of the studies and has been able to publish our key findings in a Peer-reviewed Journal (METHODS). Dr. Andre Balla, the partnering PI's main responsibilities has already been to provide tissue samples and TMA slides for the first two tasks; and we have been able to complete the key elements of our research.

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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 cancr. 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 a 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:

1. Bagasra O. *Role of zinc in Prostate Cancer*. IMPaCT convention (DOD Special Program). Atlanta, GA Sept 6th 07. Abstract # P8-9.
2. Lewis C. O. Bagasra. *Role of HERV-W in placentation*. HBCU-UP annual convention. Charleston, SC Oct 8th 07.
3. Bagasra O. *Role of HERV-w syncytin-1 in placentation*. SC IdeA Network of BioMed Res Excellence (INBRE) annual Symposium, NIH, Charleston, SC Jan 17-18.
4. Kiran B. TAM TAM Antonio V. SISON, O. BAGASRA Hima B. TAM TAM, David JASPAN. Identification of Hepatitis C virus in Cervico-Vaginal Secretions of Infected Women. Int Conf on Women's health. Dallas, TX March 2008.
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.
6. Bagasra O. *Why we do not have HIV vaccine yet?* Caglieri School of Medicine. May 20th 08. Caglieri, It.
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 Athene, Greece, Abst # PP5A-1, June 28-July 3rd. 08.
8. Bagasra O. "*Downregulation of HIV by homologous miRNAs*". AIDS2008, Mexico. August 3-6, 08.

9. Johnson LA, K Berry, O. Bagasra. *Health disparities among African Americans: the role of zinc in pathogenesis of prostate cancer*. AACR: The Science of Cancer health Disparities. Abstract # B55. Feb 3-6, 2009.
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
11. Leslie A. Johnson, Kendall Williams, Keaira Berry, Jacob Sterling, Andre' Kajdacsy-Balla and Omar Bagasra. *Differential Expression of ZIP1 in nonmalignant prostate tissue: Racial Differences*. 3rd Annual Prostate Cancer Conference, March 14-16, 2010, Atlanta GA.
12. Kanak 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.
13. Keaira Berry, Omar Bagasra, MD, PhD, Leslie Johnson. *The Role of Zinc in the Early Detection of Prostate Cancer.* Claflin University Export Program. July 11, 2008.
14. Keaira Berry, Omar Bagasra, MD, PhD, Leslie Johnson. *The Role of Zinc in the Early Detection of Prostate Cancer.* Annual Biomedical Orlando, Florida November 5- 8, 2008 Abstract B71. Page 137. Research Conference for Minority Students (*Won ABRCMS Presentation Award for 2008).
15. Keaira Berry, Bethany McGonnigal, James Padbury MD. *Placental LAT-1 Expression in Healthy and Adverse Pregnancies* Abstract G3. Page 364 Annual Biomedical Research Conference for Minority Students Phoenix, Arizona November 4- 7, 2009.
16. 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.* 3rd Annual James E. Clyburn Lecture Series University of South Carolina, Columbia April 9, 2010.
17. 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.
18. Bianca Thomas, Jazzmine Clemons, Kendall Williams, Leslie A. Johnson, Joseph P. Pestaner, Omar Bagasra. *Differential expressions of zinc transporters in β-cells in African Americans.* 3rd Annual James E. Clyburn Lecture Series University of South Carolina, Columbia. April 9, 2010.
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).
20. Jessica Abercrombie, Leslie A. Johnson, Kendall M. Williams, and Dr. Omar Bagasra. *The Molecular Relationship between Zinc and the Breast Epithelium: African Americans vs other Racial Populations*. *2nd* Annual Open House & Research Day. Claflin University, Orangeburg, SC March 27, 2010.
21. Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Kendall Williams, Joseph P. Pestaner, & 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. Pestaner, & 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.
24. 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: evaluating the molecular pathogenesis of health disparities in breast cancer.* The 5th Annual Texas Conference on Health Disparities. Fort Worth, TX May 27-28. 2010. Abstract #132.
25. Noorali, S, O. Bagasra. The role of microRNA in relationship to the protection against the human Papillomaviruses. The 5th Annual Texas Conference on Health Disparities. Fort Worth, TX May 27-28. 2010. Abstract #120.
    Re: Abstract # 285
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 to the differential expression of hZIP1.* Third AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Miami, FL Sept 30-Oct 3rd. 2010 (Abst # 285).
27. Clara L. Jones, Leslie A. Johnson, Joseph P. Pestner, & Dr. Omar Bagasra. *Zinc transporter activation and the later age of lactation may increase the risk for breast cancer.* Third AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Miami, FL Sept 30-Oct 3rd. 2010 (Abst # 140).

28. Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Omar Bagasra. *Molecular Pathogenesis of Breast Cancer: Differential Expressions of Zinc Transporters in Breast Tissues from Various Ethnic Groups*. ABRCMS 2010. Charlotte, NC
29. Sian Ramlal, Dr. Samina Noorali and Dr. Omar Bagasra. *Silencing of HLA-Transcripts expression via RNAi in Jurkat T Cell Lines.* ABRCMS 2010. Charlotte, NC
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*. ABRCMS 2010. Charlotte, NC
31. Bianca Thomas, Jazzmine Clemons, Leslie A. Johnson, and Dr. Omar Bagasra. *The Role of Zinc and Zinc transporters in the Pathogenesis of Diabetes Mellitus: Health Disparity addressed on the Bases of Race and the Environment*. ABRCMS 2010. Charlotte, NC.
32. Dr. Bagasra presented a paper at the AACR: The Science of Cancer Health Disparities. (Abstract # B55 on Feb 3-6, 2009) and travel to Carefree, AZ

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

Per Reviewed Publications:

Leslie A. Johnson, Mazhar A. Kanak, Andre' Kajdacsy-Balla, Joseph P. Pestaner, and Omar Bagasra. Differential Zinc Accumulation and Expression of human Zinc Transporter 1 (*hZIP1*) in Prostate Glands. **Methods. 2010 Aug 9. [Epub ahead of print]**

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

1. Hakim S.T., M. Al sayari, A. K. Chaitanya D. C. McLean**, O. Bagasra.** 2008. A large number of the primate MicroRNAs target lentiviruses, RE and endogenous retroviruses. **Biochemical Biophysical Research Communication** (**BBRC), 369; 357–362.**

2. **Bagasra, O**. 2008. In situ polymerase chain reaction and hybridization to detect low-abundance nucleic acid targets. ***Curr. Protoc. Mol. Biol.* 82:14.8.1-14.8.**

3. Pace G, **O. Bagasra**. 2008. NACO and the World Bank are correct in their crackdowns. **Nature Medicine**.**14: 588.**

4. Mahalingam K.**, O Bagasra**. 2008. Bioinformatics Tools: Searching for Markers in DNA/RNA Sequences. Biocomp. Vol II {g 612-615. **Proceedings of Computer Science Computer Engineering and Applied Computing**" July 14-21. Los Vegas, NV.

5. Pace DG, **O. Bagasra.** 2008**.** NACO must stop the phoney NGOs. **Indian J Med Res.** 128:87-8.

6. Addanki KC, Pace DG**, O Bagasra.** 2008**.** A Practice for All Seasons: Male Circumcision and the Prevention of HIV Transmission. **Journal of Infection in developing countries (JIDC). 2:328-334.**

7. Noorali S, ST Hakim, D McLean, SU. Kazmi, **O Bagasra.** 2008. Prevalence of Hepatitis B virus genotype D in females in Karachi, Pakistan: a review of 180 cases. **Journal of Infection in developing countries (JIDC)** 2(5): 373-378.

8. **Bagasra O**. DG Pace. 2008. Standing on the Shoulders of a Giant: Reflections on Dr. Montagnier's Nobel Prize for the Discovery of HIV-1. **JIDC** 2(6): 479-482.

9. Noorali, S., Rotar IC, C Lewis, JP Pestaner, DG Pace, A Seson and **O Bagasra. 2009.** Role of HERV-W *syncytian-1* in placentation and maintenance of human pregnancy. **Applied Immunochem & Molecular Morphology.** ; 17(4):319-28.

10. **Bagasra O**. Inaugural Editorial. **Ibnosina Journal of Medicine and Biomedical Sciences**. July 2009; 1:1-2.

11. **Bagasra. O**, Pace DG. 2010. A New Perspective on HIV Vaccine Design: A View Point**. Ibnosina Journal of Medicine and Biomedical Sciences**. 2:1-13.

12. **Bagasra O,** Pace DG. 2010. Reevaluating HIV Vaccines (submitted for publication).

13. Kanak MA, Alseiari MA, Addanki KC, Aggarwal M, Noorali S, Kalsum A, Mahalingam K, Panasik N., Pace DG, and **Bagasra O**. 2010. Triplex Forming microRNAs Form Stable Complexes with HIV-1 provirus and Inhibit Its Replication. Appl Immunohistochem Mol Morphol. May 24. [Epub ahead of print].

14. Hakim ST, Noorali S, Bagasra A., Kazmi SO, **BagasraO.** 2010. Co-infection of Hepatitis B and Hepatitis C Genotypes among Adult Population of Karachi, Pakistan (submitted to **JECH**).

15. Knight K., Addanki K., **Bagasra O**., and Kelly JF. False Positive Equals False Justice: Evaluation of the Field Marijuana Tests Used by US law Enforcement Officers in the US. (submitted for publication)

16. Noorali S., Pace, DG, and **Bagasra O**. Of Lives and Livers: Emerging Responses to the Hepatitis C Virus (Accepted for publication: **JIDC**).

17. **Bagasra O,** DG Pace. 2010. Back to the Soil: Retroviruses and Transposons. In Biocommunication of soil-bacteria and viruses (In Press). Guenther Witzany, Ed. Chapter 6.

Publications from UIC: The following publications have resulted from the current DoD CDMRP funded award.

1) Andrey J. Sarafanov, Todor I. Todorov, José A. Centeno, Virgilia Macias, Weihua Gao, Wei-Min Liang, Craig Beam, Marion A. Gray, André A. Kajdacsy-Balla. *Prostate cancer outcome and tissue levels of metal ions*. Poster presented at the United States & Canadian Academy of Pathology (USCAP) Annual Meeting, Boston, MA, April 2009. Published in **Modern Pathology** 2009 Supplement issue.

In this work studying 80 subjects, PSA biochemical recurrences after prostatectomy is associated with low zinc levels in the prostate away from tumor. This manuscript is ready to be sent out for publication, pending administrative approval from the institution of some of the collaborators (Armed Forces Institute of Pathology).

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 at UIC:

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.

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.*

Appendices: One

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Methods

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Differential zinc accumulation and expression of human zinc transporter 1 (*hZIP1*) in prostate glands

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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, and muscular) 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 (ISRTPCR) 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, and (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.

1. Introduction

The role of Zn, its underlying active function in the development and progression of prostate malignancy and its potential application in the prevention and treatment of prostate cancer are critical issues for the medical/scientific community and the public-at-large [1]. Prostate cancer (PC) is the second most common form of cancer diagnosed in men [1,2] and it is the most prevalent type of cancer observed in African American (AA) men [3–5]. Very early detection is the key to effective treatment of PC and to the prevention of deaths due to the progression of untreatable advanced stages of cancer. Mitigating factors, especially benign prostatic hyperplasia (BPH), result in a low accuracy (about 60%) of prostate-specific antigen (PSA) testing. Thus, there is an urgent need for a more reliable biomarker to identify PC at a very early stage and to identify 'at-risk' individuals. AAs are at significantly higher risk of developing PC particularly at an earlier age and have a more aggressive form of PC than European American (EA) men [1,2]. Currently, there are no satisfactory ways to differentiate between Stages I/II indolent and lethal (aggressive) PC at diagnosis. Both early identification and indolent/lethal differentiation are critical because PC, if identified while confined to the prostate, (a) is "curable" for aggressive tumors by surgery and subsequent treatment and (b) "watchful waiting" would be appropriate for indolent tumors [1–3].

Numerous studies consistently have shown that the Zn levels of malignant prostate tissue are significantly decreased as compared to the normal prostate tissue (reviewed in [6,7]). This consistency persists in different reports by different investigators employing different populations and tissue samples and involving various stages of malignancy. The studies of Zaichick et al. [8], Vartsky

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et al. [9], and Franklin et al. [7] further reveal the critically important relationship that, in individual analyses, malignant prostate tissue always exhibits relatively low Zn levels as compared to the normal tissues. In addition, Habib [11] reported that the decrease in Zn occurs early in malignancy. These persistent results, and the additional corroborating evidence presented previously (reviewed in [6,7]), has firmly established that the unique zinc-accumulating capability of the normal peripheral zone secretory epithelial cells is lost in the neoplastic transformation to malignant cells [6,7,12–14].

This study was done in order to determine if Zn molecules are differentially accumulated in various cell types and *hZIP1* is the major Zn transporter that regulates Zn in prostate glands, as previously proposed [7,15]. In order to determine if Zn accumulation in the glandular portions of the prostate is significantly different than the Zn in the stromal and other cell types, we evaluated prostatic resections from 19 men with prostate cancer and four normal prostatic tissues and evaluated their tissues for the differential Zn accumulation by utilizing two Zn indicator dyes: New Port Green DCF (NPG) and *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ) to determine the relative Zn concentrations in various histological cell types, *in situ*. In addition, we also explore the differential expression of *hZIP1* by *in situ* RT-PCR method (ISRTPCR) using the same group of patients.

2. Methods and materials

2.1. Human subjects and study protocol

Fresh frozen specimens of primary prostate carcinoma were received from the University of Illinois, Department of Pathology (Dr. Balla) on dry ice, according to the approved protocol of the institutional review board as Claflin University IRB. Normal prostate tissues were autopsy specimens obtained from the Department of Pathology, Brody School of Medicine, East Carolina University, Greenville, NC (Dr. Pestaner).

2.2. In situ RT-PCR of human tissue sections

Fresh frozen sections from 19 male prostate biopsies with a clinical history of prostate cancer, and 6 from autopsy specimens with normal glands who died from automobile accidents, were processed for RT-*in situ*-PCR [16,17]. All reagents for the continuation of the experiment were prepared in RNase-free H_2O. Briefly, fresh frozen tissue sections were received from each of our collaborators in a blinded fashion. All slides were fixed in 2% paraformaldehyde solution, overnight for a 24 h period. After fixation, slides were washed in 3× PBS once, and then twice in 1× PBS. These slides were then treated with proteinase K (6 μg/ml) at room temperature for 23 min. Proteinase K was inactivated by incubating slides on a heat block at 95 °C for 2 min. To perform the amplification of mRNA sequences for *hZIP1*, we used multiple spliced sequences that flank the junctions of two exon splice sites. Because these RNA-specific primers will not amplify the genomic DNA template, one can perform the amplification of multiple mRNAs simultaneously. The following primer pairs were used for amplification: sense 5′-TCAGAGCCTCCAGTGCCTGT-3′ and antisense 5′-TTGTCT CCTGGACCTGCTGC-3′ for *hZIP1* that gave a 189-bp product. To form cDNA copy of the template and to amplify, we used one-step *in situ* RT-PCR enzyme – *rTth* enzyme, which has both the RT and polymerase function. The amplification cocktail contained the pair of primers at 100 pM each in 50 mM Tris, pH 8.3, 8.5 mM MgCl, 10 mM $MnCl_2$, 40 mM KCL, 1 mM dithiothreitol, 10× transcription buffer, 10× chelating buffer, 5 U *rTth* (recombinant thermostable DNA polymerase) enzyme. After amplification, slides were washed in 2× SSC buffer, three times and then amplicons were detected by *in situ* hybridization method [16,17].

Hybridizations were performed with 41 bp FITC (fluorescein isothiocynate) oligonucleotide probe for *hZIP1* (oligonucleotide: 5′-FITC-CCTGGTGCCCATCTGTGTGCTGCGC-CGGCCAGGAGCTAAC-3′). Hybridizations were performed in a buffer containing 50% formaldehyde, 10 mM dithiothreitol, 2× sodium chloride/sodium citrate solution, 100 μg/mL fragmented salmon sperm DNA, 2% bovine serum albumin, 1 mg/mL *Escherichia coli* tRNA, and 20 pmol probes at 95 °C for 2 min, then 45 °C for 18 h. These tissue sections were then washed to remove unbound probes and viewed under UV-epifluorescence microscope after the cells were washed. To preserve the intensity of the hybridized probes, the tissues were not counter-stained. Parallel hematoxylin and eosin-stained slides were used to identify various histologic cell types in the tissue sections. Since we utilized frozen section the H&E parallel sections are not as crisp and sometimes difficult to identify due to tissue damage during cutting. Microscopic examination usually reveals cytoplasmic staining for mRNA versus nuclear staining for DNA. Cell enumeration was performed on coded slides by at least two pathologists. Twenty microliters of RT (reverse transcriptase enzyme) cocktail was added to each slide. The slides were then sealed with slide frame sealer and inserted into the slide slots of a thermocycler specially designed for *in situ* PCR (MJR/Bio-Rod – Twin Tower, PTC 200, Waltham, MA). The two cycles were programmed for 30 min at 50 °C, then 95 °C for 5 min (for cDNA step), and then cDNAs were amplified for 30 cycles at 95 °C denaturing, 61.7 °C annealing, and 72 °C extension.

2.3. Determination of intracellular zinc content

The relative intracellular Zn content *in situ* was determined by utilizing fresh frozen tissues. For this purpose the cells must be biochemically active. The relative concentrations of Zn in various cell types of the prostatic tissues were determined according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, Oregon, USA). Briefly, the frozen tissues were incubated with equal molar concentrations of two zinc-indicator dyes: NPG and TSQ [18,19]. The frozen tissues were incubated in 20 μl/section of the Zn indicator cocktail over night and washed in PBS, gently, without disturbing the tissues. The slides were heat-fixed for 10 s at 104 °C to immobilize the signals. These slides were mounted with solution containing 50% glycerol in PBS and observed under a fluorescent microscope. TSQ has a selective high affinity for Zn (K_d ~10 nM) and a detection limit of ~0.1 nM. The ZN-TSQ positive cells stain red [18]. NPG has moderate zinc-binding affinity (K_d ~1 μM: 19). The ZN-NPG positive cells appear yellowish green. Together, TSQ and NPG provide a relative difference in Zn concentrations in various cell types of the prostate. TSQ has about 2–3-log higher affinity for Zn than NPG, but has detection limit of about 3-log lower than NPG. Therefore, the cells that contain very low concentrations of intracellular Zn appear red and the ones with higher concentrations appear green. The cells that fall in between appear yellowish or yellow-green.

3. Results

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, Fig. 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

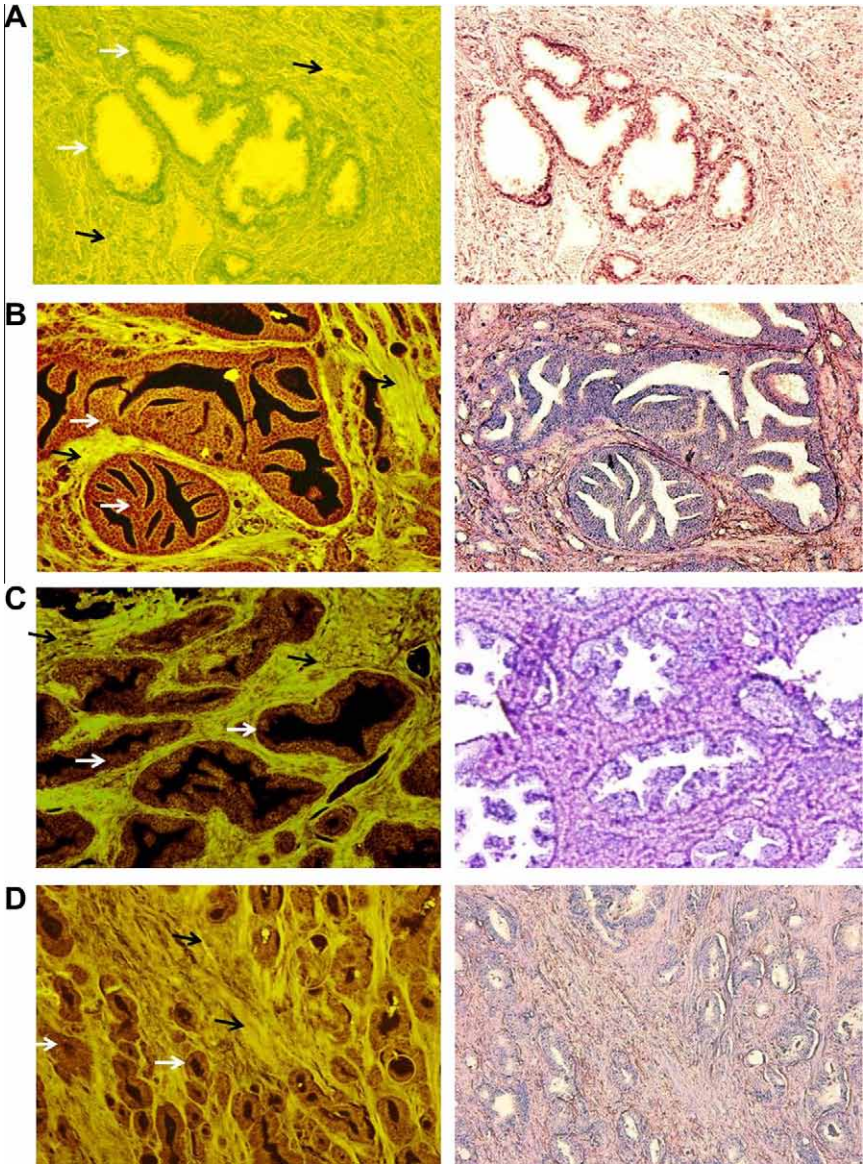

Fig. 1. Zinc levels in prostate tissue frozen sections. Representative zinc levels in prostate sections. Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. High Zn is represented by Newport Green, yellow/green stain and low Zn is represented by TSQ red stain. (A) 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 (NPG) Zn indicator dye that characterizes the normal glandular epithelial cells (black arrows, in A). 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 reductions of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are shown in (B) 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 (D) pattern is also seen in patient with adenocarcinoma Gleason score 3 + 3 (moderately differentiated) in (D). H&E sections from parallel sections are shown on right side of the slide (final magnification 100×).

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 Fig. 1B and C. Similar pattern is also seen in patient with adenocarcinoma Gleason score 3 + 3 (moderately differentiated) in Fig. 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, Fig. 2 the relative expression of mRNA expression for *hZIP1* were determined in the 19 prostate resections. The typical results represented in Fig. 2 were consistently observed in the frozen sections of all 19 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

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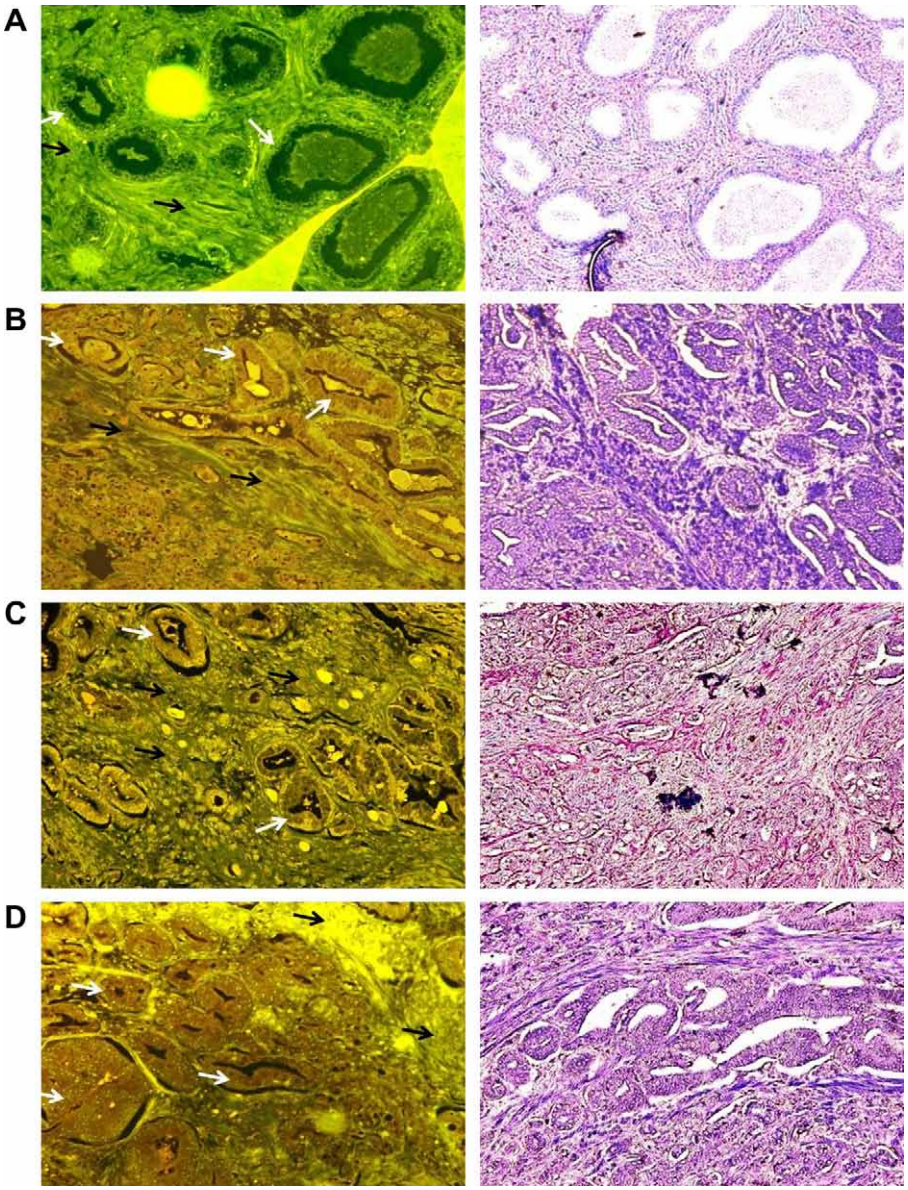

Fig. 2. (A–C) *In situ* detection of *hZIP1* mRNA expression levels in frozen prostate sections from one normal (Fig. 1A) and three prostate cancer subjects (B–D) are shown. (A) Relatively high expression of *hZIP1* in a normal prostate section, both in the glandular as well as in the stromal areas. This figure shows the relative degree of expression of *hZIP1* as determined by *in situ* RT-PCR/hybridization method. As one notes that in all three specimens from malignant tissues (B–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 magnification 100×).

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).

Fig. 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 Fig. 3A and B, 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 (Fig. 3B) as one moves further to the left (Fig. 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.

4. Discussion

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]. The

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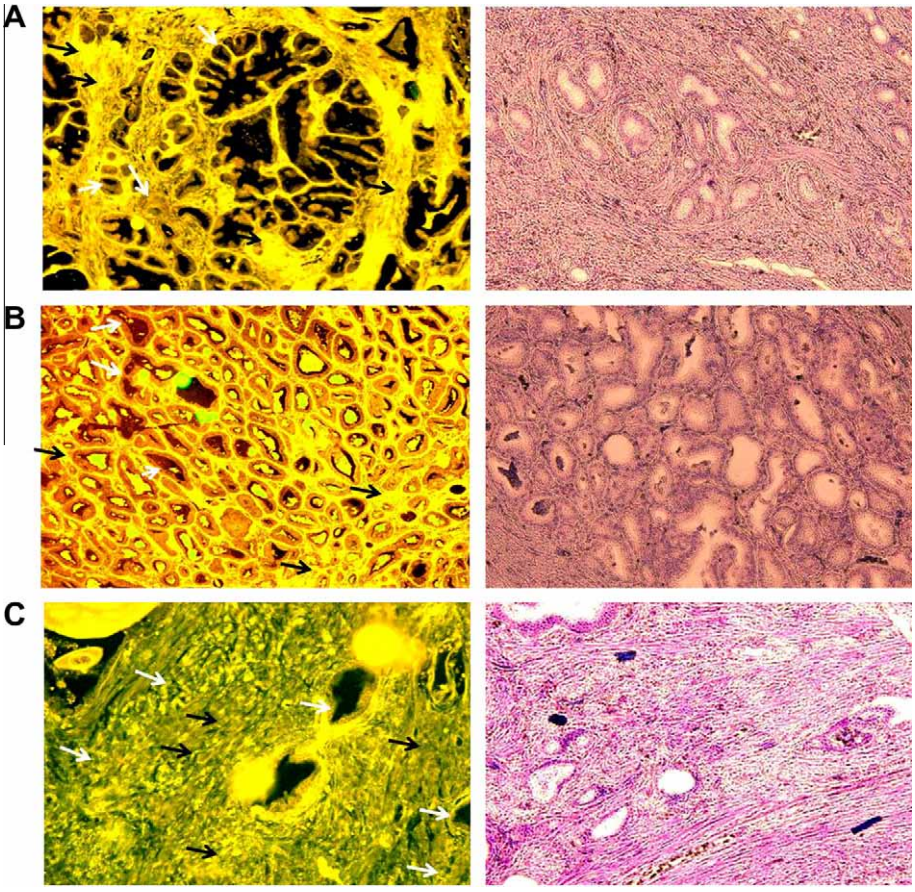

Fig. 3. *In situ* detection of *hZIP1 mRNA* and the frozen sections of normal and malignant glands in the same tissue sections. (A and B) Analyses of *hZIP1* expression by *in situ* RT-PCR/hybridization are shown from two patients. Relatively high levels of *hZIP1* expressions in normal appearing glands 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 (A) and (B) are absent, suggesting low expression of *hZIP1*. In the same patient (B) as one further moves to the left (C) 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 (final magnification 100×).

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].

4.1. 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 10-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 (nmol/g wet weight) of a normal peripheral zone tissue approximates 3000–4500; 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 decreasing 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 down-regulated [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 *hZIP1 in situ* by ISRTPCR, we demonstrate that Zn is depleted from the neoplastic as well as pre-neoplastic prostatic glandular epithelial cells. Correspondingly, *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 *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 *hZIP1* in a much larger group of patients and also show that Zn accumulation is very low in the adenocarcinomatous glands.

4.2. Significance of the methods

In the present studies, we present a new method to observe the relative Zn levels *in situ* in tissues. This method, either alone or in conjunction with *in situ* hybridization method, can be used in many different fields of science including: geomedicine (the science dealing with the influence of natural factors on the geographical distribution of problems in human and veterinary medicine: [41]), nutrition and human health research [42], in phytoremediation [43], toxicology [44], in ecological research [45], in nanotechnology [46], in environmental science to detect and to determine the remediation efforts [43,45,47,48], and in many other situations where differential detection of Zn may be important. When combined with *in situ* hybridization, the researchers can potentially uncover the molecular mechanisms of certain diseases where the relative Zn accumulation may be an important factor in the pathogenesis of many diseases and disorders (i.e. diabetes, and breast cancer) [49,50].

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