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Award Number: DAMD17-02-1-0233

TITLE: Role of Zinc in the Pathogenesis of Prostate Cancer

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REPORT DATE: September 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-09-2005	Final	26 Apr 2002 – 30 Aug 2005

4. TITLE AND SUBTITLE

Role of Zinc in the Pathogenesis of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0233

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Omar Bagasra, M.D., Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Claflin University
Orange burg, SC 29115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT – SEE ATTACHED PAGE

Incidence rates of prostate cancer are higher in blacks than in any other racial group. Our laboratory is attempting to decipher the environmental and molecular mechanisms involved in the development of prostate cancer in blacks. It is hypothesized that Africans may have genetically down-regulated their zinc absorption capacity; otherwise, they would absorb abnormally high levels of zinc, resulting in various serious neurodegenerative disorders. We hypothesized that people of African origin may have a lower capacity to transport zinc when compared with other racial groups because of their inherent down-regulation of zinc transporters. This notion was tested by evaluating 58 prostate cancer tissues in 2 major racial groups (30 from whites and 28 from blacks) for their ability to express 2 major human zinc transporters, *hZIP1* and *hZIP2*. In all 30 prostate cancer specimens obtained from white people, the degree of expression of these 2 zinc receptors was high when compared with age-matched specimens obtained from blacks. These data have been confirmed in much larger groups by utilizing zinc indicators that measure the relative intracellular zinc levels in various histological cell types of prostate tissues.

15. SUBJECT TERMS
Prostate Cancer, Zinc Transporters, hZIP1and hZIP2, gene expression

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	93	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Why are African American men, after 10 to 20 generations of residing in the U.S., twice as likely to develop cancer of the prostate as European American men, while South African Blacks are 10 to 30 times less likely to develop prostate cancer than their American distant cousins, and 2 to 10 times less likely than African Whites presumed to have a mixed European ancestry somewhat similar to that of European Americans (EA)? Are there any consistent differences in the expression of significant genes or proteins in the prostate cancers taken from African Americans (AAs) *versus* those from 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 (i.e. Asians, Native Americans, Africans or Alaskans).

In this research project, it was our goal to test this hypothesis by analyzing prostate cancer tissues for the expression of mRNAs that code for various zinc transporter proteins. Our goals are to: 1) determine the expression levels of all two zinc *transporters (hZIP1 and hZIP2*) in the neoplastic prostates from AAs *verses* EAs by utilizing the semi-quantitative reverse-transcriptase (RT) polymerase chain reaction PCR (RT-PCR), RT *in situ*-PCR, and immunocytochemistry methods, 2) measure the expression levels of the two zinc transporters (*hZIP1and hZIP2*) in the normal prostatic tissues from AAs *verses* EAs, 3) measure the intracellular zinc levels and the location of zinc inside the various cell types that make up the cancer and normal prostate tissues, and examine in a few specimens the gene sequences of zinc transporters and their promoters, which presumably regulate the degree of expression of these genes, and 4) evaluate the zinc, testosterone and prolactin levels in the blood samples of over 200 individuals from all major races, especially in AAs *verses* EAs. If a direct or environmental link between Zinc transport and prostate cancer can be established, then a special nutritional formula, medication, or other intervention might be especially designed to test the ability to decrease the incidence of this disease in AAs. Such an intervention, if successful, might be useful for persons of all populations.

In the United States, prostate cancer is the most commonly diagnosed male cancer and the second leading cause of all male cancer deaths. African-Americans have the highest prostate cancer incidence rates in the world. Our laboratories are attempting to decipher the environmental and molecular mechanisms involved in the development of prostate cancer, with special emphasis on the disproportionately high incidence rates in AAs. It is hypothesized that since Africa is a mineral-rich continent and the zinc levels in the water and diet are very high,

Africans may have genetically down-regulated their zinc absorption capacity; otherwise they would absorb abnormally higher levels of zinc, which reportedly results in serious neurodegenerative and biochemical disorders. Therefore, individuals of African origin may have a lower capacity to absorb zinc due to their inherent down- regulation of zinc transporters when compared to other racial groups. Extensive research has shown that the low serum levels of zinc have been associated with the increased incidence of prostate cancer. Our laboratories have been collaborating with the Cleveland Clinic Foundation, the Medical College of Wisconsin, and the Medical Examiner's Office of the State of Maryland to determine the degrees of expression of various zinc transporters at the molecular level. Therefore, we have evaluated 58 prostate cancer tissue samples in 2 major racial groups (30 from Caucasians and 28 from African-Americans) for their ability to express two major human zinc transporters, *hZIP1* and *hZIP2*. In all of the 30 prostate cancer specimens obtained from Caucasian individuals, the degree of expression of these two zinc receptors was higher when compared to age matched and the tumor grade level score matched specimens obtained from African-American patients. We also found significant down-regulation of these two zinc transporters in normal prostate tissues from African-American men as compared to age matched Caucasian men. When compared with normal prostate tissues, the expression levels of the zinc transporters were relatively lower in the neoplastic tissues from both racial groups tested. The loss of a unique capability to retain normal intracellular levels of zinc may be an important factor in the development and progression of prostate cancer. However, there are several questions that need to be answered before a firm correlation can be established between the actual intracellular zinc levels in the prostate glands of various racial groups and the incidence rate of prostatic neoplasm. In addition, a causal relationship needs to be determined between the zinc levels in the prostate and the levels of expression of zinc transporters, in situ. Therefore, in order to answer these questions at the molecular and cellular levels, our goals were to examine the prostate tissues and sera from the corresponding patients and explore the following **Specific Aims**:

1) To determine the expression levels of all three zinc *transporters (hZIP1 and hZIP2*) in the neoplastic prostates from AAs verses EAs, by utilizing the quantitative reverse-transcriptase (RT) polymerase chain reaction PCR (RT-PCR) method, RT *in situ*-PCR, and immunocytochemistry. This project has been **completed** and expanded. Please see **Appendices I, II and III**

2) To measure the expression levels of all two zinc transporters (*hZIP1and hZIP2*) in the normal prostate tissues from AAs verses EAs (Completed: Appendices I, II and III,).

3) To measure the blood zinc levels in about 2,000 individuals and compare the serum zinc levels in African-Americans, Africans, Caucasians, Asians and mixed racial groups. These studies will be performed at the Principal Investigator's site-Claflin University--a HBCU. For this purpose, the majority of the serum specimens were to be received from the Medical University of South Carolina (MUSC). However, the investigator moved to UAB and was unable to provide the already collected specimens. Therefore, we initiated our own specimen collection and have now received around two hundred serum/plasma specimens. We are standardizing the methods and will measure the specimens for zinc contents. In addition to zinc levels, we will also measure the testosterone and prolactin levels in these groups (data are being collected and analyses). For the last eight months we have also received around 1,100 specimens from Dr. Harold W. Lischner, who retired in 2004 and donated a large number of specimens for Dr. Bagasra. We intend to analyze these specimens also after we standardize our assays. In order to assist us in our analyses, we have recruited Dr. Kalapathy, an Associate Professor in the Department of Chemistry. He is an expert in trace metal measurements. His CV is attached. This work will need few at least six months to complete.

4) In a separate but smaller group of individuals, autometallography and atomic absorption spectrophotometry will be used to measure intracellular zinc in various anatomic parts of the prostate tissues. We have developed a better and more robust method for intracellular measurement of Zinc in situ (see below for details). We have developed a differential staining methods that can recognize low and high amounts of intracellular zinc (please see **Appendix IV. (Manuscript in Press)**

5) To determine which other factors, including the exposure to prolactin, testosterone, external zinc concentrations, and combinations of these three agents regulate the zinc transporters in the pre-established prostatic cell lines (i.e. PC-3 and La cell lines) and primary cell lines established from the prostate tumors from various racial groups. We believe that by establishing a link between the low intracellular transport capacity of zinc in the African-American population and development of prostate cancer, we may be able to design protocols which can increase intracellular zinc levels in the prostate gland. In addition, we hope to identify certain unique genes that may be selectively expressed or suppressed in certain racial groups. These studies may also shed some light on why men from all races develop prostate cancer in old age and how it is linked to intracellular zinc levels and serum zinc, testosterone, and prolactin levels (preliminary work has been **completed** and was presented at the 96th Annual meeting of AACR: see attachments). **Appendices V and VI.**

6) **Role of Zinc in Diabetes**: Since, we have hypothesized that one of the major reason AAs disproportionally suffer from prostate cancer is due to their inherent inability to transport appropriate amount of zinc in the crucial cell types that require relatively higher amount of zinc than the other cell types. It would be logical to explore that this possible genetic and environmental link between human zinc transporters (hZIPs) and their differential expressions in the islet beta cells from AAs as compared to other racial groups, particularly EAs, in both normal healthy individuals and diabetic patients would yield some information. We hypothesize that the hZIPs play an important role in the development of diabetes, and the main reason AAs disproportionately suffer from DM (as well as other illnesses like prostate and pancreatic cancers, hypertension, and CVD) as compared to EAs may be due the low degree of expressions of the critical zinc transporters in the b cells. Understanding the molecular events in the pathogenesis of DM with regards to regulation of zinc uptake would be critical to the evaluation of the natural history of diabetes in humans and especially in various racial groups. If a direct link between zinc transport and diabetes can be established, then a special nutritional formula, medication or other intervention might be especially designed to test the ability to decrease the incidence of this disease in DM susceptible groups, particularly in AAs. This argument is presented in **Appendix VII**, published in Medical Hypotheses in August 2005).

Establishment of New Collaborations: We have now collaborated with two other research centers (Department of Biomedical Sciences, Dental School. University of Maryland, Baltimore, MD, and Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY), and have independently analyzed the prostate tissues for hZIP expression and for the intracellular levels of zinc in the normal verses malignant tissues. The results of our studies have been submitted for publication and are summarized below.

We now show that gene expression of ZIP1 is down regulated in adenocarcinomatous glands along with a decline in the cellular level of zinc. Evidence indicates an epigenetic silencing of ZIP1 gene expression in the development of prostate malignancy; and implicates *ZIP1* as a tumor suppressor gene. This study supports and extends the concept of the role of zinc and altered metabolism in the pathogenesis of prostate cancer. **Please see Appendix IV for complete details.**

Determination of intracellular zincs concentration by zinc indicators. Total intracellular cellular zinc concentrations ***in situ*** can be measured in the live cells and fresh frozen tissues using the zinc-indicator dyes Newport Green DCF, PDX and TSQ (Molecular Probes, Eugene, OR). As opposed to autometallography, the zinc indicator can provide relative intracellular zinc levels in various anatomical portions of the prostate sections from same individual as well as

allows for the comparative analyses of intracellular zinc in the prostates from different racial groups that are age-matched.

Zinc concentrations in the 1–100 nM range can be measured using fluorescent indicators more recently developed indicators with greater Zn^{2+} selectivity (39-49). We have focused our development efforts on probes for detection of higher Zn^{2+} concentrations that are present in prostate peripheral zone (PZ). Peak concentrations of intracellular Zn^{2+} in the PZ of a Caucasian man may exceed 100 μM (unpublished data and please see photos above). We are currently assessing the value of the recently developed FuraZin-1 (F24182, F24183), IndoZin-1 (I24184, I24185), FluoZin-1 (F24180, F24181), FluoZin-2 (F24188, F24189) and RhodZin-1 (R24186, R24187), a series of unique indicators designed for detection of Zn^{2+} in the 0.1–100 μM range with minimal interfering Ca^{2+} sensitivity from Molecular Probes (50-62). The spectral responses of these indicators closely mimic those of the similarly named Ca^{2+} indicators. For instance, FuraZin-1 (57) and IndoZin-1 (44) exhibit Zn^{2+}-dependent excitation and emission spectral shifts, respectively; FluoZin-2 (45) and RhodZin-1 46 (47) show Zn^{2+}-dependent fluorescence without accompanying spectral shifts.

Newport Green DCF and Newport Green PDX Indicators

Since the beginning of 2004 we are evaluating Newport Green DCF indicator (Molecular Probes, N7990, N7991) that has moderate zinc-binding affinity (K_d for Zn^{2+} ~1 μM) but is essentially insensitive to Ca^{2+} (K_d for Ca^{2+} >100 μM), making this a valuable probe for detecting intracellular Zn^{2+} in various portions of the prostate gland. When used alongside dyes with dual Ca^{2+}/Zn^{2+} sensitivity such as fura-2 and mag-fura-2, Newport Green DCF provides confirmation that changes in Zn^{2+} levels, and not Ca^{2+} or Mg^{2+}, are being detected (59-61). Newport Green PDX [48] (N24190, N24191) incorporates the same di-(2-picolyl)amine chelator as Newport Green DCF (Figure 1 above) but has a higher Zn^{2+} dissociation constant (K_d for Zn^{2+} ~30 μM) and a larger Zn^{2+}-free to Zn^{2+}-saturated fluorescence intensity increase. Newport Green DCF has been used to identify insulin-producing β-cells from human pancreatic islets on the basis of their high intracellular Zn^{2+} content (53).

TSQ

Use of the membrane-permeant probe *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ, M688) in cells was first described by Fredrickson (58). TSQ is selective for Zn^{2+} in the presence of physiological concentrations of Ca^{2+} and Mg^{2+} ions (59). The complex of TSQ with free Zn^{2+} apparently has a stoichiometry of two dye molecules per metal atom (62-69), but a 1:1 complex

may be formed with metalloproteins. The intracellular Zn^{2+} chelator dithizone blocks TSQ binding of Zn^{2+} (58, 63).

Several reports suggest that TSQ can be used to localize Zn^{2+} pools in the central nervous system (54-56). Zn^{2+} moves from presynaptic nerve terminals into postsynaptic nerve terminals when blood flow is constricted in the brain. This translocation is reported to correlate with ischemia-caused neurodegeneration, as determined by the fluorescence of TSQ (65). TSQ has also been used to detect nitric oxide–induced accumulation of free Zn^{2+} in neuronal perikarya (57) and changes in Zn^{2+} distribution in the rat hippocampus and amygdale (58) during and after kainic acid–induced seizures. TSQ (like Newport Green DCF) is a selective nontoxic stain for pancreatic islet cells, which have a high content of Zn^{2+}, and may be useful for their flow cytometric isolation (59-62).

TSQ-based assays for Zn^{2+} in seawater and other biological systems exhibit a detection limit of ~0.1 nM (58, 62-65). The simultaneous determination of Zn^{2+} and Cd^{2+} by spectrofluorometry using TSQ in an SDS micelle has also been reported (64). TSQ has been used to measure Zn^{2+} levels in artificial lipid vesicles and live sperm cells by flow cytometry (60).

Recently, we have acquired 60 frozen prostate tissue section from our collaborator at the Medical College of Wisconsin (35 from Caucasian and 25 from AAs) and are evaluating the intracellular Zn+ levels in these tissues. The initial work has been completed but histological examination and data analyses will take several months to complete. After our analyses we will send these data to our collaborator and he will break the code and determine the accuracy of our initial finding. **This work is being prepared for publication.**

KEY RESEARCH ACCOMPLISHMENTS

- We began with a crucial question: Why are African American men, after 10 to 20 generations in the U.S., twice as likely to develop cancer of the prostate as Caucasian Americans, while South African Blacks are 10 to 30 times less likely to develop prostate cancer than their American distant cousins and 2 to 10 times less likely than African Whites, who may be presumed to have a mixed European ancestry somewhat similar to that of White Americans?

- In order to answer this question in a most definitive fashion, we have divided our possible answers into various categories. The first and foremost of the question was to determine if there are any consistent differences in the expression of

significant genes or proteins in the prostate cancers taken from AAs versus those from White Americans. 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 factor worthy of epidemiological research for its potential relationship to the incidence or progression of prostate cancer in AAs, EAs, or other groups. For this purpose we chose to evaluate the relative degree of expression of human zinc transporters crucial for retaining the zinc into the prostate.

- We have evaluated 58 prostate cancer tissues in 2 major racial groups (30 from EAs and 28 from AAs) for their ability to express 2 major human zinc transporters, *hZIP1* and *hZIP2*. In all 30 prostate cancer specimens obtained from White people, the degree of expression of these 2 zinc receptors was high when compared with age-matched and Gleason score-matched specimens obtained from African American patients.

- We also found a significant down-regulation of these two zinc transporters in normal prostate tissues from African American men when compared with age-matched White men.

- We have began to set up the highest state of the art methods to determine the intracellular levels of zinc in the 60 frozen specimens collected until this date. Our goal has now measured the intracellular zinc levels and the location of zinc inside the various cell types that make up the cancer and normal prostate tissues. Four undergraduate minority students have completed their analyses in the PI's laboratory, and these students will be presenting the results of their studies at the 96th Annual Meeting of AACR (during April 2005). The copies of Abstracts are included as **Appendices V and VI**).

- We trained a total of eight undergraduate minority students to carry out tissue culture methods on well-defined prostate cancer cell lines. These cell lines were analyzed for the relative expression of zinc transporters before and after the exposure to various concentrations of zinc, testosterone, and prolactin. Results are included as **Appendices V and VI.**

- The blood samples that were supposed to come from MUSC will not be available due to the relocation of our collaborator to UAB. We initiated collaborations with

two local clinics in Orangeburg. In addition, we have developed a formal collaboration with a clinical group in Virginia, which have provide the blood specimens we need to carry out our studies. We collected over 200 blood specimens from all major races to evaluate concentrations of zinc, testosterone, and prolactin in AAs versus EAs. These studies are in progress.

- We are certain that there is a direct link between zinc transport and prostate cancer. If a strong link is established between the environment, genes, and diet, then a special nutritional formula, medication, or other intervention might be especially designed to test the ability to decrease the incidence of this disease in AAs. Such an intervention, if successful, might be useful for the persons of all populations.

REPORTABLE OUTCOMES:

Manuscript (published):

Rishi I, Baidouri H, Abbasi JA, Bullard-Dillard R, Kajdacsy-Balla A, Pestaner JP, Skacel M, Tubbs R, **Bagasra O**. Prostate cancer in African American men is associated with downregulation of zinc transporters. Appl Immunohistochem Mol Morphol. 2003 Sep;11(3):253-60.

Bagasra, O. 2004. Citrate Sours the Malignant Intent of Prostate Epithelia (editorial) **Mitochondria** 4:339-341.

Costello, LC, R.B. Franklin, P Feng, M Tan, **O. Bagasra**. 2005. Zinc and Prostate Cancer: A critical scientific, medical and public interest debate! Cancer Causes and Control (2005) 16:901–915.

R. B. Franklin, P. Feng, B. Milon, M. M. Desouki, K. K. Singh, A. Kajdacsy-Balla, **O. Bagasra,** L. C. Costello. Down Regulation of Expression of hZIP1 Zinc Uptake Transporter and Depletion of Zinc in Prostate Cancer. A Possible Tumor Suppressor Gene (In Press: Molecular Cancer)

Abstracts: The following Abstract has resulted from this award:

Student Presentations and Abstracts (Students names in **red**):

Anita Carter & Omar Bagasra. Role of Zinc Indicators in the Intracellular Determination of Zn+ in Normal and Malignant Portions of Prostate Gland. SC Life Colloquium of Undergraduate Research. April 3, 2004.

Paula D. Perry, Omar Bagasra. Detection of SV 40 Gene Sequences in the Non-Hodgkin's Lymphoma Tissues. At the annual Biomedical Research Conference for Minority Students (ABRCAMS) Nov 10-14, 2004. Dallas, TX, USA. Abstract #C75..

Dyantha Moody, Meaghen Ashby, Naomi Kariuki, Jenica Hemingway, Frank Gooden, Andre' Kajdacsy-Balla and Omar Bagasra. 2005. Effects of various steroid hormones on intracellular zinc uptake in prostate cancer cell lines. 96th Annual Conference of AACR Abstract # 3204. April 2nd to 6th 2005.

Stacy Gilliard and Omar Bagasra. 2005. Differential expression of various zinc transporters in human prostate cancer. 96th Annual Conference of AACR Abstract # 3235. April 2nd to 6th 2005.

Presentation: The PI presented data on prostate cancer at the following locations:

- Bagasra, O. New Frontiers in Morphology. 10th International Conference of molecular morphology. Oct 5-8, 2002. Santa Fe, NM.
- Bagasra, O. A New Idea in Prostate Cancer Prevention. Invited speaker at San Francisco State University. San Franciasco, CA. May 22, 2003.
- Bagasra O. RNAi: a new revolution in molecular biology. Annual Meeting of the Am. Society of Investigative Pathology. Session 275. Trends in Experimental Pathology: Frontiers in Molecular Morphology Translational Research. Wash. D.C., April 18th 2004. FASEB Pg 103-104. April 17-21, 2004.

Patents and licenses applied for and/or issued: A patent is being prepared on the effects of zinc supplement in the prevention of Prostate Cancer in the African American Population.

Degrees obtained that are supported by this award:

Nine minority students worked on this project as part of the requirement of their undergraduate degree:

- Tiffany Brown (currently in Med school: Ohio Univ)
- Melodie Harrison (currently in Med School; MUSC)
- Anita Carter (currently in graduate School: Univ of Pittsburg)
- Paula Perry (applying for Med School)
- Meaghen Ashbey (current student)
- Dyntha Moody (current student)
- Jenica Hemingeway (current student)
- Frank Gordon (summer intern: UMD)
- Ashly Joe (currently in training)

Development of cell lines, tissue or serum repositories; infomatics such as databases and animal models, etc. None

Funding applied for based on work supported by this award:

An application was submitted to NCI based on the preliminary result from this award and was funded. The details are as follows;

FUNDED BY NCI

NCI: 08/01/2003 to 04/31/06 "Training grant for Claflin University Students"
In collaboration with USC Cancer Center
$566,035/yr with USC Cancer Center, P.I.: O. Bagasra

PENDING at DOD

DOD PC040560 10-1-2004 to 9-31-2007 $566,000 for 3 yrs.
Role in the Project: PI
"MOLECULAR TARGETS FOR ZINC IN PROSTATE CANCER PREVENTION"

See Summary Statement from the Reviewers (we received an Excellent Score). See **Appendix PCO4050**

Employment or research opportunities applied for and/or received based on experience/training supported by this award. YES!

CONCLUSIONS: We started our project to address a very important question concerning why African American men, after 10 to 20 generations in the U.S., twice as likely to develop cancer of the prostate as Caucasian Americans, while South African Blacks are 10 to 30 times less likely to develop prostatic cancer than their American distant cousins and 2 to 10 times less likely than African Whites, who may be presumed to have a mixed European ancestry somewhat similar to that of White Americans. Are there any consistent differences in the expression of significant genes or proteins in the prostate cancers taken from African Americans versus those of White Americans? 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 groups.

We have concluded that there are significant differences between EAs and African Americans with regards to the degree of expression of two zinc transporters that are involved in importing zinc from the outside into the prostate glands. There are many additional assays that need to be performed. We feel that intracellular zinc levels by Zn+ indicators would provide a strong support for this hypotheses, if Zn+ levels in the malignant Verses Normal and African American Verses Caucasians support our initial report. Once these data are confirmed in larger groups, this finding could have a significant application as a preventive maneuver at least in African Americans. Because dietary zinc supplements are relatively nontoxic, any efficacy trial would be low-risk.

Also, African Americans disproportionately suffer from various diseases in the US. Many of these diseases include hypertension, lupus, cardiovascular disease, diabetes mellitus, and cancers of the prostate and pancreas. A number of risk factors such as smoking, a high fat diet, little physical activity, stress, and meager access to health care have been the subject of numerous investigations. However, the factor of the interaction between genetics and the environment has received very little attention in the basic scientific community. This work is being carried our currently in the PI's laboratory and a manuscript is included as Appendix II for the review.

According to numerous epidemiological data in the 21st century, patients suffering from DM will increase more than in the 20th century. For those reasons, the creation and

development of a new class of pharmaceuticals for the treatment of DM in the 21st century is extremely desirable. In the last half of the 20th century, investigations between the relationships among diseases and micronutrients, such as iron, copper, zinc, and selenium, have been numerous.

Our laboratory is investigating the potential role of zinc transporters in the pathogenesis of many illnesses that disproportionately affect the African American community.

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Applied Immunohistochemistry & Molecular Morphology 11(3): 253–260, 2003

Prostate Cancer in African American Men Is Associated With Downregulation of Zinc Transporters

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In the United States, prostate cancer is the most commonly diagnosed male cancer and the second leading cause of all male cancer deaths. Furthermore, incidence rates are higher in African Americans than in any other racial group. Our laboratory is attempting to decipher the environmental and molecular mechanisms involved in the development of prostate cancer in African Americans. Because Africa is a mineral-rich continent, and the zinc levels in the water and diet are high, it is hypothesized that Africans may have genetically downregulated their zinc absorption capacity; otherwise, they would absorb abnormally high levels of zinc, resulting in various serious neurodegenerative and biochemical disorders. It is therefore possible that people of African origin may have a lower capacity to absorb zinc when compared with other racial groups because of their inherent downregulation of zinc transporters. Extensive research has shown that low serum levels of zinc are associated with the increased incidence of prostate cancer. We have evaluated 58 prostate cancer tissues in 2 major racial groups (30 from whites and 28 from African Americans) for their ability to express 2 major human zinc transporters, *hZIP1* and *hZIP2*. In all 30 prostate cancer specimens obtained from white people, the degree of expression of these 2 zinc receptors was high when compared with age-matched and Gleason score-matched specimens obtained from African American patients. We also found a significant downregulation of these 2 zinc transporters in normal prostate tissues from African American men when compared with age-matched white men. The loss of the unique ability to retain normal intracellular levels of zinc may be an important factor in the development and progression of prostate cancer. Our observation that the uptake of zinc may be different in racial groups is intriguing and relevant. Once these data are confirmed in larger groups, this finding could have significant application as a preventive maneuver for at least for some people. Because dietary zinc supplements are relatively nontoxic, any efficacy trial would be low-risk.

Manuscript received February 24, 2003; accepted March 4, 2003.

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Supported in part by a grant from the Department of Army: DAMD 17-02-1-0233.

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Applied Immunohistochemistry & Molecular Morphology 11(3): 253–260, 2003.

The prostate contains high amounts of free zinc ions that are excreted into the seminal fluid. The extracellular and intracellular distribution of zinc ions in the rodent using highly specific autometallographical studies have shown that zinc accumulates primarily in the acinic lumen of the lateral lobes, whereas the dorsal lobe stains only faintly and the ventral lobe is void of grains.[1,2] At the ultrastructural levels, the presence of zinc ions is confined to apical secretory vesicles and the epithelium of mainly the lateral lobes in both rodents and humans.[1,3] Recently, Iguchi et al,[4] using semiquantitative reverse-transcriptase polymerase chain reactions (SQ-RT-PCRs), showed that the expression of zinc transporter (ZnT) 2 in rats was very high in the lateral and dorsal prostate and much lower in the ventral prostate. In humans, it appears that zinc ions are constantly secreted from the epithelial cells into both the acinic lumen and the intercellular canaliculi.[5] Prostate secretory epithelial cells have the unique function and capability of accumulating extremely high intracellular levels of zinc.[2–5] One of the effects of this accumulation is the inhibition of cell growth, partly because of an increase in apoptosis. The accumulation of high intracellular levels of zinc by prostate cells induces mitochondrial apoptogenesis.[6] Prolactin and testosterone regulate zinc accumulation in the prostate; however, little information is available concerning the mechanisms associated with zinc accumulation and its regulation in prostate epithelial cells.[7,8] By using the human malignant prostate cell lines LNCaP and PC-3, Costello et al[7] have shown that the zinc accumulation in both cell types is stimulated by physiologic concentrations of prolactin and testosterone. Their studies reveal that these cells possess the ability to uptake zinc rapidly, indicative of the presence of a plasma membrane high-affinity zinc transporter, possibly by the regulation of the ZIP-type zinc transporter gene expression.[7,8] Kinetic studies demonstrate that the rapid uptake of zinc is effective under physiologic conditions that reflect the

total and mobile zinc levels in circulation.[8] Correspondingly, genetic studies demonstrate the expression of a ZIP family zinc uptake transporter in both LNCaP and PC-3 cells.[8] Some of these zinc-accumulating characteristics are found to be specific for prostate cells. These studies support the concept that prostate cells express a unique hormone-responsive, plasma membrane-associated, rapid zinc uptake transporter gene that is associated with their unique ability to accumulate high zinc levels.[9–11]

In the United States, the incidence of prostate cancer is significantly higher in African Americans than in white people or Asian Americans.[12–19] We hypothesized that because Africa is a mineral-rich continent and zinc levels are relatively high in the water and diet, abnormally high amounts of zinc in the blood may result in various neurologic and metabolic abnormalities. The zinc absorption and transport systems are genetically downregulated in the African populations. The phenomenon may be similar to sickle cell anemia, in which a single mutation has provided the survival advantages against the ravages of the fatal form of malaria.[20] We hypothesize that when African people entered North America, mostly during the slave trade, they encountered an environmental problem: in North America, the zinc levels are relatively low, and the native populations (the American Indians) and other races who migrated from Europe and Asia have a higher capacity to transport zinc to various organs, especially to the prostate gland, in an appropriate manner. This genetic regulation can be compared with the expression of melanin pigments in the white and black populations.[21] Here the situation is reversed. The white population is unable to upregulate their melanin pigmentation sufficiently, even in the presence of strong sunlight, to protect them from damaging solar ultraviolet (UV) light. Squamous cell carcinoma is the most common tumor of sun-exposed epithelium in white populations.[21] Therefore, just as white people carry an evolutionary disadvantage against the solar UV rays outside of their ancestral low-UV light environment, the low absorption capacity of zinc has created a disadvantage in the peoples of African descent who have migrated outside Africa. If this is the case, long-term low serum concentrations of zinc deprive the prostate gland of its essential source of vital trace mineral ingredients, resulting in prostate metaplasia and neoplasia. A large body of the scientific literature and careful studies support the idea that low levels of zinc contribute to the high incidence of prostate cancer.[1–10]

In this research study, it was our goal to test this hypothesis by analyzing the prostate tissues for their ability to express 2 major zinc transporters responsible for accumulation of zinc in the prostate glands. For this purpose, we used a highly sensitive RT-in situ-PCR method to compare the relative levels of expression of the 2 zinc transporters, *hZIP1* and *hZIP2*, in 2 racial groups in the United States (white and African American).

METHODS AND MATERIALS

Human Subjects and Study Protocol

Archival, formalin-fixed, paraffin-embedded specimens of primary prostate carcinoma were retrieved from the files at the Department of Pathology of the Medical College of Wisconsin. Similarly, fixed tissues from radical prostatectomy specimens were obtained from the Cleveland Clinic Foundation, according to the approved protocols of the respective institutes. Normal prostate tissues were autopsy specimens obtained from the State of Maryland Medical Examiner's Office at Baltimore, Maryland.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction Zinc Transporters

The total mRNAs were harvested from the deparaffinized tissues as described previously.[22–24] The RNA preparations were used to quantitate the levels of zinc transporters, and the relative levels of expression were visualized according to each racial subgroup.

Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction

The details of the SQ-RT-PCR have been described previously.[22–24] The major advantage of this protocol that allows the relative quantitation of each of the specific RNA species in the samples is the use of standard curves of in vitro transcribed mRNAs of *hZIP2*. Plasmids containing full-length *hZIP2* and pCMV-hZIP2 were kindly provided by Dr. David J. Eide of the Department of Nutritional Sciences, University of Missouri. This clone was used to develop a standard curve to semiquantitate the relative degree of the expression of *hZIP2*. The full-length *hZIP2* shows a significant homology to the members of the *ZIP* family, including *hZIP1*. Therefore, we were able to design primer pairs that could amplify the conserved sequences of hZIP1 and hZIP2 by multiplex polymerase chain reaction. The concentrations of the mRNAs were measured spectrophotometrically, and the relative copy numbers of mRNAs present in 1 μL of solution were calculated using the molecular weight of the transcript and the Avogadro number. Relative numbers of each *ZIP* were derived by SQ-RT-PCR and generated by a serial 2-fold dilution of *pCMV-hZIP2* plasmid DNA. In the linear amplification range of these curves, the copies of in vitro transcribed mRNAs were plotted against the relative size of the amplified bands of the amplified fragments of the 2 *hZIPs*. Using these dilution curves of the plasmid (performed in duplicate), the relative number of the spliced mRNAs for *hZIP1* and *hZIP2* were calculated. All samples were tested in at least 3 independent experiments. As a control, we performed quantitative RT-PCR of β-actin, as we described previously.[22–24]

Reverse-Transcriptase In Situ Polymerase Chain Reaction

Paraffin sections from 58 prostate biopsies of men with clinical histories of prostate cancer, and 4 from autopsy specimens of people with normal glands who died of automobile accidents, were processed for RT-in situ-PCR. Briefly, paraffin-embedded tissue sections of the specimens were received from each of our collaborators in a blinded fashion. All the reagents were prepared in RNase-free reagents. Therefore, all the slides were deparaffinized with sequential washings with EM-grade xylene, absolute alcohol, 95% ethanol, and 70% ethanol for 5 minutes each, then washed twice in a 1× phosphate-buffered saline (PBS). After deparaffinization, these slides were further fixed in a Streck fixative (STF; Streck Labs, Inc, Omaha, NE) for 2 hours. Incubating slides in 3× PBS for 10 minutes inactivated STF. The slides were washed twice in 1× PBS. These slides were treated with proteinase K (6 μg/mL) at room temperature for 22 minutes. Proteinase K was inactivated by incubating slides on a heat block at 95°C for 5 minutes. To perform the amplification of mRNA sequences for *hZIP1 and hZIP2,* we used multiply spliced sequences that flank the junctions of 2 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: sense 5′-ACCAGACAAGGAC-TTCA-ATTAC-3′ and antisense 5′- GAGGACTAAAGCTGA-AAACATC-3′ for *hZIP1*, and sense 5′-GAATCACAG-ATTCAGAAGTTCA-3′ and antisense 5′-CTCTCCAT-AGGGATACTC CATA-3′ for *hZIP2*. The amplification of β-actin mRNA was performed by using a pair of primers: 5′-ATCTGGCACCTTCTACAATGAGCTGCCG-3 and 5′-CGTCATACTCCTGATTGCTGATCCACA-CATCTGC-3′. The *hZIP2* gave a 102-bp product and *hZIP1* gave a 189-bp product. The β-actin pair yielded 838-bp amplicons.[22–24] To amplify, we used the *rTth* enzyme, which has both the RT and polymerase function.[23] The amplification cocktail contained the pair of primers at 100 pM each in 50 mM Tris pH 8.3, 8.5 mM $MgCl_2$, 10 mM $MnCl_2$, 40 mM KCL, 1 mM dithiothreitol, 10× transcription buffer, 10× chelating buffer, 5 U *rTth* recombinant thermostable DNA polymerase) enzyme, and 200 mM of each deoxyribonucleoside triphosphate. Twenty μL 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-Twin Tower, PTC 200, Waltham, MA). The 2 cycles were programed for 30 minutes at 62°C, the 94°C for 2 minutes (for cDNA step), and then cDNAs were amplified for 30 cycles at 92°C denaturing, 55°C anneaing, 72°C extension.

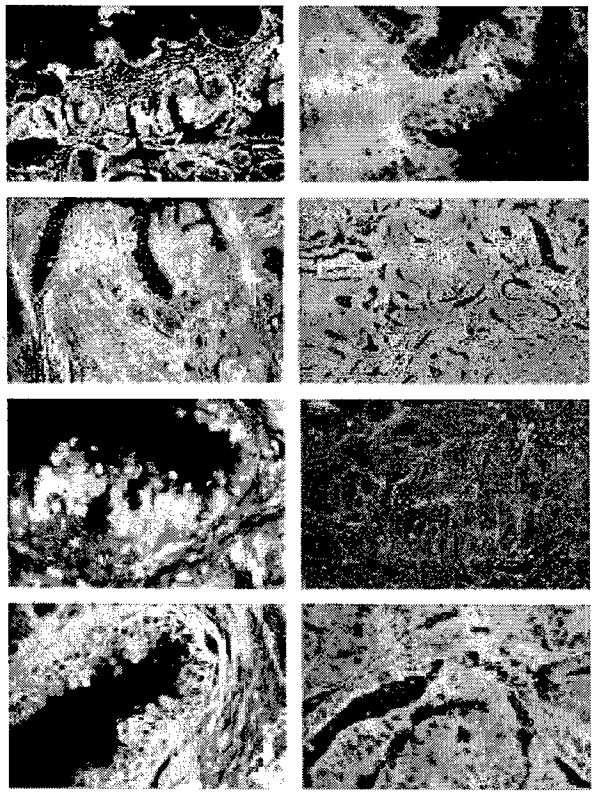
Hybridizations were performed with either Cy3 = 2-amino-3-mercaptopropionamide or an FITC (fluorescen isothiocynate) oligonucleotide probe for the *hZIP1* and *hZIP2* sequences (oligonucleotide: 5′-CAGC-AAGTGAGAGAAATTCTTCTGGTGATGCTGATTC-AGCTC-3′ and 5′-CTTAGAATTTCAGTGGAGTC-TTTTTCCTCTTGCAGTTTAAAGCAAAAGTC-3′). 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 minutes, then 40°C for 18 hours. These tissue sections were then washed to remove unbound probes and viewed under UV epifluorescence microscopy 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. Microscopic examination usually reveals cytoplasmic staining for mRNA versus nuclear staining for DNA.[22–24] Cell enumeration was performed on coded slides by at least 2 pathologists.

RESULTS

Degree of hZIP1 and hZIP2 Expression in the Malignant Prostate Tissues From White and African American Men

We evaluated the *hZIP1* and *hZIP2* expression, the 2 major zinc transporters, by simultaneously performing a multiplex RT-*in situ*-PCR. We evaluated 58 prostate cancer specimens in a blinded manner for their level of expression of these 2 zinc transporters. The majority of the specimens were from patients who had a 3+3 or 3+4 Gleason score. Upon unlocking the blinded codes, all the specimens from the white men exhibited a significantly higher degree of expression of the 2 zinc transporters than the majority of the specimens from the African Americans. Therefore, all 30 specimens from the white men's prostate biopsies exhibited a modest degree of expression of both of the zinc transporters, whereas 26 of 28 prostate specimens from the African Americans exhibited a low or very low expression of both the zinc transporters. In 1 of the other 2 specimens from African Americans, the prostate sections exhibited high expression of *hZIP1* and low expression of *hZIP2*, whereas in the second case, a reverse pattern of expression was observed. In Figure 1, we have shown representative microphotographs of 8 prostate intraepithelial lesions from age-matched specimens, 4 from each racial group.

Figure 2 shows high-grade prostate carcinomas from 6 age-matched specimens, 3 from each racial group. As it is evident in Figures 1 and 2, the degree of expression of both zinc transporters is significantly higher in the prostate tissues from white men than in the age-matched



Caucasian men
Green = *hZIP2*
Red = *hZIP1*

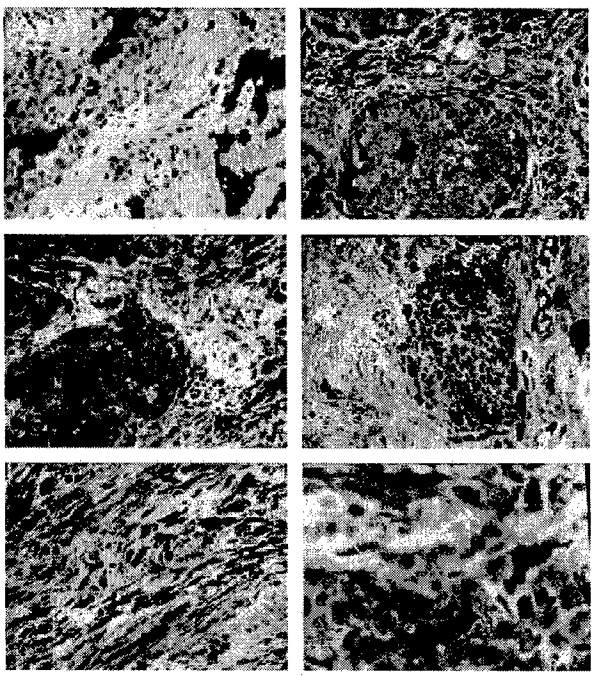
African American men

FIGURE 1. Representative photomicrographs showing the expression of 2 zinc transporters in intraepithelial lesions, *hZIP1* and *hZIP2*, by multiplex RT-in situ-PCR. Four intraepithelial neoplastic lesions from white men (left) and age-matched specimens from African American men (right) are shown. The coexpression of the zinc transporters, *hZIP1* (red) and *hZIP2* (green), the neoplastic lesions and areas surrounding the tumor lesions exhibit a wide variation in the coexpression of the zinc transporters, *hZIP1* (red) and hZIP2 (green), in the white group. In African Americans, the prostate cancer from the tumors and surrounding areas exhibited a markedly decreased expression of the zinc transporters compared with the tumors from the white patients.

lesions from the African Americans. The coexpression of the zinc transporters *hZIP1* and *hZIP2* in the neoplastic lesions and areas surrounding the tumor lesion showed a wide variation in the expression of these zinc transporters in all tissues from both races. However, with the exception of 2 cases, we observed a consistent decreased degree of the expression in prostate tissues from African Americans over that seen in their counterparts, regardless of their tumor grade. The prostate cancer from the African Americans' tumors and surrounding areas exhibited a markedly decreased expression of zinc transporters compared with the tumors from the white patients. Of note, in all cases, the expressions of both the zinc transporters were visibly lower in the neoplastic areas compared with the surrounding normal-appearing areas. This finding is consistent with the data indicating that overall, the zinc levels are lower in the malignant portions of the prostate gland.[3]

We also performed SQ-RT-PCR analyses in the mRNAs isolated from the prostate tissues of the 8 men shown in Figure 1 for *hZIP1*, and also from 6 people for hZIP2, shown in Figure 2. As shown in Figure 3, the relative expression in the specimens from African Americans of both zinc transporters, *hZIP1 and hZIP2*, were significantly lower when compared with their age-matched counterparts.

To demonstrate that mRNAs were not degraded in the paraffinized prostate specimens, we analyzed the presence and integrity of β-actin mRNAs by RT-PCR. RT-PCR for β-actin was performed on all the specimens. As shown in Figure 3, 8 specimens evaluated for *hZIP1* also had intact mRNAs for β-actin, which clearly demonstrate the integrity of mRNAs in the specimens we analyzed for the ZIP transporters. More importantly, there was no significant difference in the degree of amplification between the specimens isolated from white men and



Caucasian men
Green = *hZIP2*
Red = *hZIP1*

African American men

FIGURE 2. Representative photomicrographs showing the expression of the 2 zinc transporters in high-grade tumors, *hZIP1* and *hZIP2*, by multiplex RT-in situ-PCR. Shown are 6 intraepithelial neoplastic lesions, age-matched specimens, 3 from each racial group: white (left) and African American men (right). In African Americans, the prostate cancer from the tumors and surrounding areas exhibited a marked decrease in the expression of the zinc transporters compared with the tumors from the white patients.

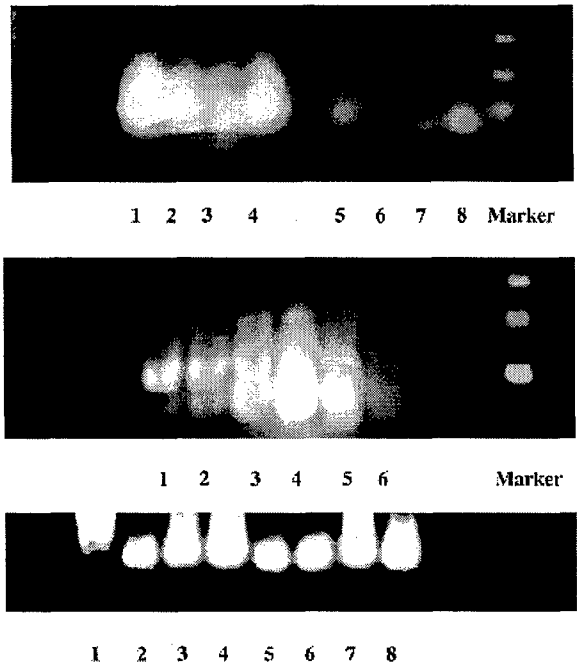


FIGURE 3. Semiquantitation of spliced mRNAs of *hZIP1* and *hZIP2* zinc transporters by QS-RT-PCR. (Top) mRNAs from each specimen were isolated from the 8 people shown in Figure 1. RT-PCR was performed. Ethidium bromide-stained gel electrophoresis of *ZIP1* shows the relative degree of the amplifications. Lanes 1, 2, 3, and 4 are from white tissues, whereas lanes 5, 6, 7,and 8 are from the specimens from African Americans. (Middle) Amplifications of *hZIP2* from the total cellular mRNA isolated from the 6 neoplastic lesions. These specimens were randomized, and RT-PCR was performed for *hZIP2* on the 6 specimens shown in Figure 2. Lanes 1, 2, and 5 are from specimens from African Americans. Lanes 3, 4, and 5 are from specimens from white subjects. (Bottom) mRNAs isolated from the 8 specimens and tested for hZIP1 (top) were also tested for the presence and integrity of β-actin mRNA. Amplification of β-actin from the total cellular mRNA was successful, and there was no significant difference in the degree of amplification between the specimens isolated from white people and African Americans.

African Americans. These analyses validated 2 important points: (1) the mRNAs we isolated were intact, and (2) the differences we observed in the expressions of the zinc transporters were not caused by relative degradation of mRNA signals in different specimens.

In 2 of 28 specimens from African Americans, we observed a significant upregulation of 1 of the 2 *hZIP* transporters, but not both. As shown in Figure 4, two prostate neoplastic lesions exhibited an overexpression of either *hZIP1 or hZIP2*, but not both. The inheritance of the overexpression of *hZIP1 or hZIP2* could have resulted from the interbreeding that occurred in past generations between the white and African American parents (or grandparents) of these people. This could have resulted in the correction or overcorrection of the genetic downregulation of the *hZIP1* or *hZIP2* transporters. These observations also point toward a promoter-mediated regulation of these 2 zinc transporters. This possibility is currently being evaluated in our laboratory.

To determine whether the relatively low expression of *hZIP1 and hZIP2* in African Americans is limited only to neoplastic areas, we examined the prostate tissues from normal, nonneoplastic tissues from healthy men of both races who died because of automobile accidents. As shown in Figure 5, there is a high degree of the expression of both *hZIP1* and *hZIP2* within the normal prostate tissues from 3 normal white males, whereas the expression in the prostates of 2 healthy African Americans was, at best, moderate.

DISCUSSION

African Americans have the highest prostate cancer incidence rate in the world.[13–18,25] At a global level, the rates of incidence are low in Asian and African men, low to moderate in white men, and high in African American men.[13,25] Using data collected between 1988 and 1992, Wingo et al[14] reported that African Americans have a 35% higher incidence rate and a 223% higher mortality

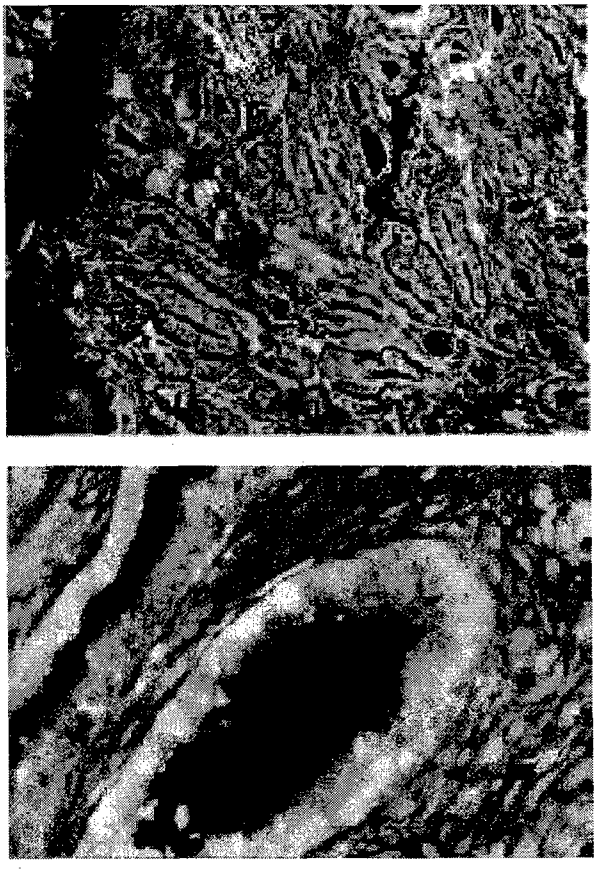
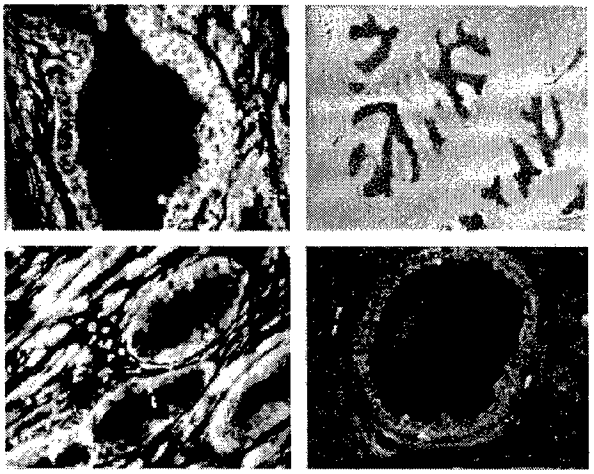


FIGURE 4. Representative photomicrographs from 2 neoplastic specimens from African American men. (Top) Upregulation of *hZIP1* (red) and relative downregulation of *hZIP2.* (Bottom) Upregulation of *hZIP2*, whereas *hZIP1* expression is almost absent.



Caucasian men
Green = *hZIP2*
Red = *hZIP1*

African American men

FIGURE 5. Representative photomicrographs showing the relative expression of 2 zinc transporters, *hZIP1* and *hZIP2*, in the normal prostate glands by multiplex RT-in situ-PCR. The relative expression of *hZIP1 and hZIP2* in 2 normal prostate tissues from white men (left) and 2 age-matched African American men. Note that there is a high degree of expression of both *hZIP1* and *hZIP2* within the normal prostate tissues from 2 normal white men, whereas the expression in the prostates of 2 normal African American men would be at best scored as moderate.

rate from prostate cancer when compared with whites. The differences in the incidence and mortality between African Americans and whites are attributed to screening, environmental, and biologic factors.[16,17] When compared with white controls, black men present at a younger age with a higher grade and stage of the disease for their age, and with a greater delay in diagnosis.[18,19] Whether the pathogenesis of prostate cancer is different in African American men compared with white men remains unanswered. Whittemore et al[18,36] note that African American men appear to have a larger volume of "latent prostate cancer." These investigators believe that larger-volume latent carcinomas are those that progress to become clinically evident at a faster rate, suggesting that the events that account for racial differences in prostate cancer incidences may occur very early in cell transformation, and thus may be genetically controlled.

We have hypothesized that the major reason for the high incidence of prostate cancer in African Americans may be their inherent inability to absorb or transport normal amounts of zinc from the Northern American environment, in which there is a relatively low concentration of zinc in the diet. We have further hypothesized that zinc absorption and transport systems are genetically downregulated in some of the African populations. Such natural selection may occur because of the serious adverse effects on the nervous system caused by high zinc levels.[26–28] However, when African people entered North America, mostly during the slave trade, they may have encountered an environmental disadvantage because of their inherent downregulation of zinc transporters. On this continent, the zinc levels are relatively low, and the native populations (the American Indians) and other races who migrated from low zinc areas, ie, Europe and Asia, have a higher capacity to absorb zinc and are able to transport it to the other organs in an appropriate manner. This genetic regulation can be compared with the expression of melanin pigments in white and non-white populations. The white population is unable to up-regulate their melanin pigmentation sufficiently, even in the presence of strong sunlight, to protect themselves from the damaging solar UV light of wavelengths between 290 and 320 nm. Skin cancer is the most common type of cancer in the United States; more than 600,000 cases of skin cancer are reported each year in this country in the white population. Squamous and basal cell carcinomas are the most common tumors of sun-exposed skin areas in this group.[21] Just as white people carry an evolutionary disadvantage against the solar UV light outside their low UV light ancestral environment, the low absorption capacity of zinc has created a disadvantage in people of African descent when they migrate outside Africa. Long-term low serum concentration of zinc deprives the prostate gland of its essential source of a vital trace mineral ingredient, resulting in prostate metaplasia and neoplasia. A large body of the scientific literature and careful studies support the association of low levels of zinc with prostate neoplasia.[3–6] Zinc is an essential nutrient to all organisms because it is a required catalytic or structural cofactor for 100s of zinc-dependent enzymes and other proteins such as transcription factors. An example of effect of low serum levels of zinc can be seen in the animal model of *lethal milk* mouse mutant.[29] ZnT4 is deficient in the *lethal milk* mouse mutant, in which pups of any genotype suckled on homozygous *lethal milk* mothers die of zinc deficiency before weaning. The zinc level in the milk of homozygous *lethal milk* animals is approximately 50% that of normal animals, demonstrating that ZnT4 plays a crucial role in development.[29] Various reports suggest that regardless of race and geographic location, the etiology of certain prostate carcinomas may be linked to zinc transporters. Because the expression of zinc transporters also appears to be regulated by prolactin and testosterone, an age-related increase in the incidence rate of this malignancy may also be indirectly linked to the zinc uptake by the prostate gland.[8–11]

Members of the *ZIP* family are found in all cellular life forms, including archaebacteria, eubacteria, and eukaryotes.[10,30–34] There are currently approximately 85 members reported in the sequence databases. These fall into 4 subfamilies based on their amino acid similarities.[10] Most members are predicted to have 8 transmembrane

domains and share a predicted topology where the amino and carboxyl termini are extracytoplasmic. There are 12 known ZIP members in the human genome.[31] Three of the human proteins, *hZIP1, hZIP2*, and *hZIP3*, are very closely related to the fungal and plant proteins known to be zinc uptake transporters. *hZIP2* expression has been detected only in prostate[31] and uterine[32] epithelial cells, suggesting that this protein plays a very specialized tissue-specific function. On the other hand, hZIP1 is expressed in all 24 human tissues examined so far.[33] Gaither and Eide[10,11,31] have sequenced and characterized the *hZIP2* gene, a human zinc transporter identified by its similarity to zinc transporters recently characterized in fungi and plants. *hZIP2* is a member of the *ZIP* family of eukaryotic metal ion transporters that includes 2 other human genes, *hZIP1* and *hZIP3*, and the genes in mice and rats.[10,11]

The human genome contains at least 3 *ZIP* family members. The current hypothesis is that these genes encode zinc uptake transporters.[10,11] We can gain some insight into *ZIP* function in humans by considering the tissues in which these proteins are expressed. Repeated attempts by Gaither and Eide[11] to detect *hZIP2* mRNA on Northern blots of poly (A)+ RNAs derived from different human tissues and cultured cell lines failed to produce positive results. It appears that the *hZIP2* transporter gene is normally expressed at low levels and in specific cell types, and that a more sensitive detection method is required. We also attempted to quantitate *hZIP2* mRNA by Northern blots; however, several attempts were not productive. Therefore, we decided to use highly sensitive RT-in situ-PCR and SQ-RT-PCR methods. Gaither and Eide[10,11] isolated only 4 *hZIP2*-expressed sequence tag clones found only in prostate and uterine cDNA libraries. The observation that these particular tissues express *hZIP2* may be instructive in that cells of the prostate contain the highest zinc level of any soft tissue in the body. Any potential downregulation in this transporter may play a pivotal role in the pathogenesis of prostate cancer. Thus, it appears that the expression of *hZIP2* in prostate and uterine tissues may help meet their particular needs of zinc metabolism. In contrast, the low-affinity *hZIP1* and *hZIP3* have been cloned as expressed sequence tags from a large number of different tissues, indicating that these genes are widely expressed and may play general housekeeping roles.[10]

Therefore, observed zinc transporter expression may be associated with the great need for zinc involved in the normal processing of the prostate gland functions, a lack of which may have caused the molecular injury resulting in the development of prostate cancer.[7–11] Low serum levels of zinc have been associated with the increased incidence of prostate cancer.[7–12] Previously, Costello et al[8,9] have shown that *hZIP1* is expressed in PC-3 cells, and that a zinc uptake was actively upregulated testosterone and prolactin treatment. Furthermore, *hZIP1* expression was regulated by zinc availability. Therefore, when PC-3 cells were exposed to high zinc, *hZIP1* mRNA levels were downregulated. The molecular mechanisms by which low zinc levels contribute to the development of neoplasia are still obscure, and limited data are available. Costello et al[9] have shown that long-term cellular zinc deficiency leads to an increase in cell proliferation partly because of a reduction in apoptosis. The accumulation of high intracellular levels of zinc by prostate cells induces mitochondrial apoptogenesis, indicating a physiologic effect of zinc in the regulation of prostate cell growth.

Thus, in prostate cancer, 2 themes emerge from the analyses of zinc transporter expression in vivo: (1) the downregulation of zinc transporters by either genetic inheritance (African descent) or through aging (related to the modulations in the testosterone/prolactin levels or gene expressions acquired with old age) leads to the low accumulation of zinc in the prostate tissues,[12–19] and (2) the loss of the unique capability to retain normal intracellular levels of zinc caused by either the increased export or low import of zinc may be an important factor in the development and progression of malignant prostate cells.[1–5,10,29–35] From our data, it appears that the lowest degree of the expression of zinc transporters, *hZIP1* and *hZIP1*, is localized in the areas that exhibit neoplastic lesions, and is less dominant in the areas that are healthy-appearing. Our observation that there are differences in the zinc transport in different racial groups has great significance for prevention. If a role of zinc transporters is clearly established, then a zinc supplementation could be helpful in at least some people. Understanding the molecular events in the pathogenesis of prostate cancer is critical to the evaluation of the natural history of prostate cancer in humans, especially in various racial groups.[34–38] □

Acknowledgments: The authors thank Dr. Eide for providing us with the plasmid with *hZIP2*. We are grateful to April J. Adams for her editorial assistance.

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Mitochondrion 4 (2004) 339–341

Mitochondrion

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Editorial comment

Citrate sours the malignant intent of prostate epithelia☆

It has been well documented that prostate epithelial cells contain high concentration of zinc and these levels are significantly decreased in prostate carcinoma relative to normal prostate tissue and are increased in benign prostatic hyperplastic (BHP tissue) (Beck et al., 2004; Boyle et al., 2003). Although zinc is essential for proper maintenance of all cells, it is particularly important in the prostate which secretes high levels of citrate and proteins that contains zinc (reviewed in Byar, 2003; Cooper and Farid, 1964). There is compelling evidence that zinc is involved in the pathogenesis of prostate cancer (Byar, 1974; Cooper and Farid, 1964; Costello et al., 2004a). Of note, the prostate gland in a human male is divided into three zones: the peripheral zone (PZ) covers about 70% of the gland, whereas the central zone (CZ) is comprised of 25% and the transition zone (TZ) covers the remainder of the 5%. Most interestingly, the majority of the prostate cancer occurs in the PZ and this area essentially looses its ability to retain zinc in the cancerous epithelial cells (Byar, 1974; Cooper and Farid, 1964). On the other hand, zinc is significantly increased in the BPH area (located in the CZ) and that portion of the glands rarely develops carcinoma (Byar, 1974; Cooper and Farid, 1964). A major function of the PZ epithelium is to secrete an extraordinary amount of citrate and the same zone accumulates about 10-fold more zinc than the rest of the gland (Byar, 1974; Cooper and Farid, 1964; Costello et al., 2004a). Importantly, the decrease in zinc and in citrate occurs early in malignancy, before any histopathological changes can be discerned under the microscope (Costello and Franklin, 1998). Zinc depletion proceeds decreased citrate production (Costello and Franklin, 1998). The question that begs the answer is, 'why the PZ epithelia of the prostate gland would accumulate such high zinc levels, particularly when it is well documented that high zinc levels can have adverse consequences at the molecular and cellular levels (Costello et al., 2004b, 1997, 1999)'? And, curiously, why the same cells that contain such a high zinc level also produce an enormous amount of citrate (Costello et al., 2004a)? It has been established that the accumulation of mitochondrial zinc inhibits m-aconitase activity and citrate oxidation (Costello et al., 2004a). Is it citrate that protects the prostate epithelial cells in PZ from the toxic effect of zinc? In this issue Costello et al. (Dineley et al., 2002), explore the effects of zinc on mitochondrial terminal oxidation. They provide excellent documentation through a series of well-designed experiments that free zinc ions would inhibit cellular respiration and terminal oxidation, and hence, a high intracellular mitochondria level of zinc in the PZ prostate cells is essential for the normal physiology of these cells (Costello et al., 2004a). The cytoplasmic zinc is transported to mitochondria via one or more intermediary molecules that subsequently inhibit the terminal step in the electron transport system. Such an inhibition significantly reduces the total energy output at the cellular levels, forcing the cells to rely on aerobic oxidation of glucose. A decrease in the zinc uptake and accumulation results in citrate oxidation and production of more energy, allowing cells to go malignant (after certain neoplastic mutations to take place). The authors hypothesize that more energy efficient specialized citrate producing epithelial cells of the PZ of the prostate may be an

☆ Refer to article DOI: 10.1016/j.mito.2004.07.031, also published in Vol. 4 (pp. 331–338).

1567-7249/$ - see front matter
doi:10.1016/j.mito.2004.08.001

essential step towards conversion from normal to malignancy step (Costello et al., 2004a; Dineley et al., 2002; Gaither and Eide, 2000).

Even though, there are still many questions that remain to be answered, the current observations reported by Costello et al. are highly relevant in the understanding of the molecular pathogenesis of prostate cancer.

The most important issues that needs to be resolved in the area of prostate cancer are that:

(1) Unlike other malignant tumors that are monoclonal in nature, prostate cancer is multi-focal (reviewed in Foster et al., 2000). Does the lost ability of PZ cells to accumulate zinc initiate the malignancy process? One can imagine that the lost ability to take in zinc would arise at multiple loci in the PZ! If so, then what are the events at the molecular levels?
(2) The significant clues regarding the uptake of zinc are already accumulating with a great speed. It is now known that there are several zinc transporters that work in concert to regulate zinc uptake. ZIP transporters, hZIP1–hZIP4, are plasma membrane proteins that belong to the superfamily of Zrt/rt-like proteins ands are involved in the uptake of zinc from the extracellular environment (reviewed in Huang et al., 2002; Kirschke and Huang, 2003; Prodan et al., 2002).
(3) Several hormones are known to up-regulate ZIP transporters, including prolactin and androgens (Beck et al., 2004; Prodan et al., 2002).
(4) Various ZIPs are differentially expressed in human prostate cells (Puttaparthi et al., 2002; Rishi et al., 2003). The factors that govern the differential expressions of these transporters may add in the therapy and prevention of prostate cancer.
(5) There is evidence that genetic factors also play an important role in the degree of expression of ZIP1 and ZIP2 transporters. These two transporters are down-regulated in malignant areas of the PZ as compared to surrounding normal cells. Most importantly, expressions of both of these zinc transporters are significantly more down-regulated in African Americans as compared to age and Gleason score-matched white men (Rishi et al., 2003). Of note, African Americans have twice as much incidence of prostate cancer than whites (Zaichick et al., 1997).

Answers to these questions may help us conquer this illness that takes millions of lives annually worldwide.

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Cancer Causes and Control (2005) 16:901–915
DOI 10.1007/s10552-005-2367-y

Zinc and prostate cancer: a critical scientific, medical, and public interest issue (United States)

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Received 26 August 2004; accepted in revised form 15 February 2005

Key words: aconitase, citrate, prostate cancer, zinc, ZIP1 zinc transporter.

Abstract

The role of zinc in the development and progression of prostate malignancy and its potential application in the prevention and treatment of prostate cancer (PCa) are contemporary critical issues for the medical/scientific community and the public-at-large. The overwhelming clinical and experimental evidence provides a compelling rational basis for the expectation and concept that prostate zinc accumulation is an important factor in the development and progression of prostate malignancy; and that zinc could be efficacious in the prevention and treatment of PCa. In contrast, various epidemiologic studies have produced divergent and conflicting results regarding the efficacy of dietary and supplemental zinc against PCa. Before reaching any definitive conclusions regarding this complex issue, one should have a complete understanding of the clinical and experimental evidence associated with the involvement of zinc in the normal and malignant prostate. Also, an understanding of interacting effects of confounding factors on the absorption, assimilation, and bioavailability of supplemental dietary zinc is important. The purpose of this review is to present the current state of the clinical and experimental information regarding zinc relationships in the normal prostate and in the pathogenesis PCa. The evidence in support of a potential beneficial effect of zinc supplement versus potential harmful effects on PCa is assessed. A discussion of the divergent results of the epidemiologic studies is presented along with a description of important factors and conditions that impact or mask the effects of dietary zinc on PCa development and progression. We also hope to bring more attention to the medical and research community of the critical need for concerted clinical and basic research regarding zinc and PCa, for the development of appropriate human prostate models to investigate these relationships, for further appropriately designed epidemiologic studies, and for future well-controlled clinical trials.

Introduction

The importance of zinc in the pathogenesis of prostate cancer (PCa) and its potential for the prevention and treatment of PCa is a critical contemporary issue for the scientific/medical community and the public-at-large. The overwhelming and consistent clinical and experimental evidence that we present in this review and in earlier reviews [1–4] provides a compelling rational basis for the expectation and concept that altered prostate zinc accumulation is an important factor in the development and progression of prostate malignancy. This evidence also provides a rational basis for the expectation that the use of supplemental zinc under conditions that would restore zinc accumulation in malignant prostate cells should be efficacious in the prevention and treatment of PCa. Despite the consistent clinical and experimental evidence for a protective effect of zinc against the development and progression of PCa, various epidemiologic studies have produced divergent and conflicting results regarding the efficacy of dietary and supplemental zinc against PCa. The epidemiologic reports range from no effect of zinc [5–7], possible beneficial effects of zinc [8, 9], and possible harmful effects of zinc [10, 11]. One should ask, "Why the

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overwhelming and consistent clinical and experimental evidence is not also reflected in a corresponding consistency in the results of the epidemiologic studies?"

A comprehensive understanding of the zinc relationships in normal prostate and in PCa as it relates to zinc in the prevention of PCa or as a risk factor for PCa is essential. A critical assessment of the existing clinical and experimental supporting evidence for the beneficial effects of zinc versus its harmful effects is an essential requisite to reaching any conclusions regarding the role of zinc in the pathogenesis, prevention and treatment of PCa. An understanding of the multivariate factors associated with the use of dietary zinc supplements and the assimilation and bioavailability of zinc is also essential. Such information is also critical to the planning of epidemiologic and clinical trial studies and the interpretation of the results of such studies. In this review we hope to provide a comprehensive review that integrates the relationships involved in the role of zinc in the development of prostate malignancy and its potential in the treatment and prevention of PCa.

Is zinc involved in the pathogenesis of PCa?

This is the first essential question that must be addressed, and it is central to the critical issue of the potential efficacy of zinc in the treatment and prevention of PCa (see [1–4] for detailed reviews of zinc relationships in normal and malignant prostate). To address this issue, one must recognize that the peripheral zone is the essential region of the human prostate gland that is involved in the zinc relationships. This is also the dominant region for the origin and development of malignancy; and our discussions will relate to this region.

Zinc levels in normal peripheral zone versus malignant tissue

It is well established that the normal peripheral zone has the function of accumulating extremely high zinc levels that are three- to ten-fold greater than found in other soft tissues (Table 1). This capability resides in the highly specialized secretory epithelial cells of the peripheral zone. In contrast, the central zone and its secretory epithelium do not exhibit this functional ability. Therefore, we characterize the peripheral zone secretory epithelial cells as "zinc-accumulating" cells. To attain this ability, these cells must possess specialized mechanisms that permit the accumulation of high cellular zinc levels. This is in contrast to the general relationship in which most mammalian cells contain processes that prevent the accumulation of high levels of reactive zinc that could be cytotoxic.

The malignant prostate cells that develop in the peripheral zone do not contain the high zinc levels that characterize the normal secretory epithelial cells. As represented in Table 2, the zinc levels of malignant prostate tissue is about 60–70% lower than the normal peripheral zone tissue. Indeed, measurements of pure malignant tissue in the absence of normal glandular epithelium would likely reveal even lower zinc levels that would approximate the levels found in other soft tissues. It is important to note the variability of the reported zinc changes in BPH versus normal prostate, whereas the reported decrease in zinc in malignant prostate is strikingly consistent. This consistency persists in different reports by different investigators employing different populations and tissue samples and involving various stages of malignancy. The study of Zaichick *et al.* [19] further reveals the critically important relationship that, in individual tissue sample analyses, no malignant sample exhibited the high zinc level that characterizes normal prostate sample (Figure 1). A similar observation is reported by Vartsky *et al.* [23]. In addition, Habib [14] reported that the decrease in zinc occurs early in malignancy. In recent studies (unpublished, manuscript in preparation), we show for the first time with *in situ* studies that the normal peripheral zone glandular epithelium exhibits high cellular zinc levels; and that the adenocarcinomatous glands exhibit a depletion of zinc (Figure 2). Moreover, the decrease in zinc is evident in low-grade and high-grade malignant glands. It is now apparent that decreased zinc levels in malignant prostate

Table 1. Representative citrate and zinc levels in prostate

	Citrate (nmols/gm wet wt.)	Zinc (nmols/gm wet wt.)
Normal central zone	4000	1000
Normal peripheral zone	13000	3000
BPH	14000	4000
PCa (malignant tissue)	500–2000	500–900
Other soft tissues	150–450	200
Blood plasma	90–110	15
Prostatic fluid	40000–150000	9000

Table 2. Zinc levels of normal prostate, benign prostatic hyperplasia, and PCa

Citation	Normal	BPH	PCa
[12]	540	746 (+42%)	168 (−69%)
[13][a]	517	460 (+11%)	194 (−62%)
[14][a]	–	531	272
[15][a]	–	845	150
[16][b]	125	760 (+508%)	46 (−63%)
[17][b]	156	234 (+50%)	39 (−75%)
[18][b]	348	774 (+123%)	79–147 (−57 to 77%)

[a] μg zinc/gm dry wt.
[b] μg zinc/gm wet wt.

tissue involves a decrease in the intracellular level of zinc in the malignant cells, which eliminates the concern that decreased secretory content, (i.e. the production of prostatic fluid), not cellular depletion, is responsible for the decline of citrate in PCa tissue samples.

This decrease in zinc, along with citrate changes described below, is the most consistent and persistent biochemical change that differentiates malignant loci from normal peripheral zone. In contrast to the many reports that demonstrate this zinc relationship, no reports (to our knowledge) exist which dispute or present opposing evidence of this zinc relationship in human prostate tissue samples. Based on these clinical relationships and the citrate relationship described below, it follows that the malignant prostate cells *in situ* have lost the ability to accumulate zinc; and that malignant prostate cells *in situ* with high zinc levels virtually never exist!

The zinc–citrate connection

Further compelling evidence is provided by the following citrate relationship in normal and malignant prostate (for reviews see [1–4]). That the normal peripheral zone has the major function of accumulating and secreting extraordinarily high levels of citrate is well established (Table 1). In contrast, the citrate levels of malignant prostate tissue are dramatically and consistently decreased. The consistency of this relationship serves as the basis for the recent development of magnetic resonance spectroscopy imaging (MRSI) for the *in situ* detection of malignant loci (Figure 3; see [24–26] for reviews). Figure 1 presents the composite results from three independent studies of *in situ* MRS determination of citrate levels in normal peripheral zone versus malignant loci. Two important points are evident: (1) the citrate levels of malignant loci are significantly lower

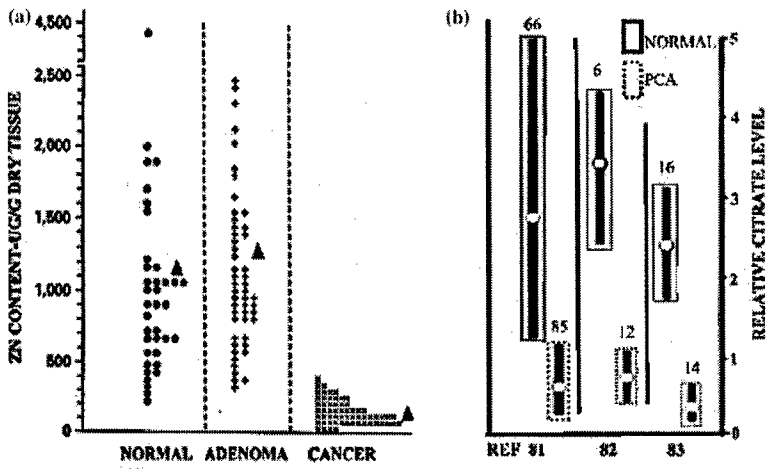

Fig. 1. Zinc and citrate levels in normal prostate, BPH, and PCa. (a) Zinc levels of resected prostate tissue. Taken from Zaichick *et al.* [19]. (b) *In situ* citrate levels determined by magnetic resonance spectroscopy. Results taken from Kurhanewicz *et al*, [20], Heerschaap *et al.* [21], and Liney *et al.* [22].

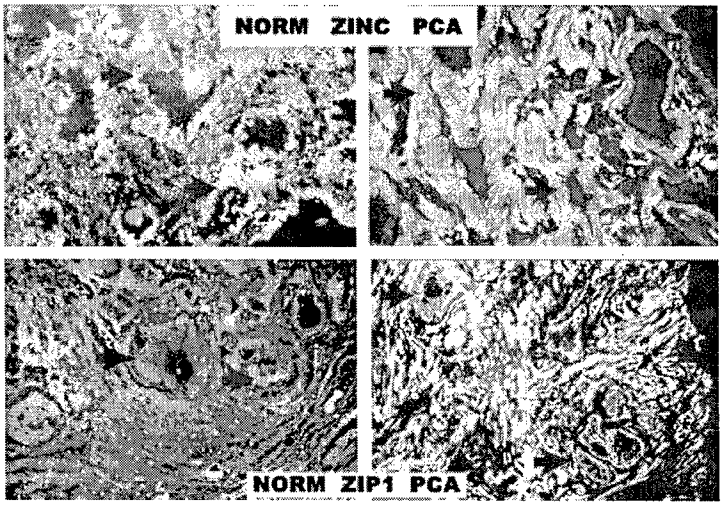

Fig. 2. The *in situ* determination of ZIP 1 expression and zinc levels in normal versus adenocarcinomatous glands. The normal glandular epithelium exhibits ZIP1 expression and high zinc levels. The malignant glands exhibit an absence of detectable ZIP1 expression and a depletion of zinc.

than corresponding normal peripheral zone; and (2) the malignant tissue never exhibits a high citrate level. Moreover, this citrate relationship also exists in malignancy associated with the transition zone [27]. In addition, Cooper and Farid [28] reported that the decrease in citrate occurs early in malignancy, which is verified by the MRS studies. The early MRS studies represented in Figure 1 have been confirmed by several more recent reports from other laboratories; and no MRS study that presents opposing results has been reported. Thus, the citrate relationships mimic the zinc relationships described above.

We have established that the zinc and citrate relationships are coupled as a "cause and effect" [29]. In normal prostate epithelial cells, the accumulation of high levels of mitochondrial zinc results in inhibition of mitochondrial (m-) aconitase activity; which prevents the oxidation of citrate, thereby leading to the accumulation of citrate (Figure 4). Changes in citrate levels are directly due to and preceded by changes in the level

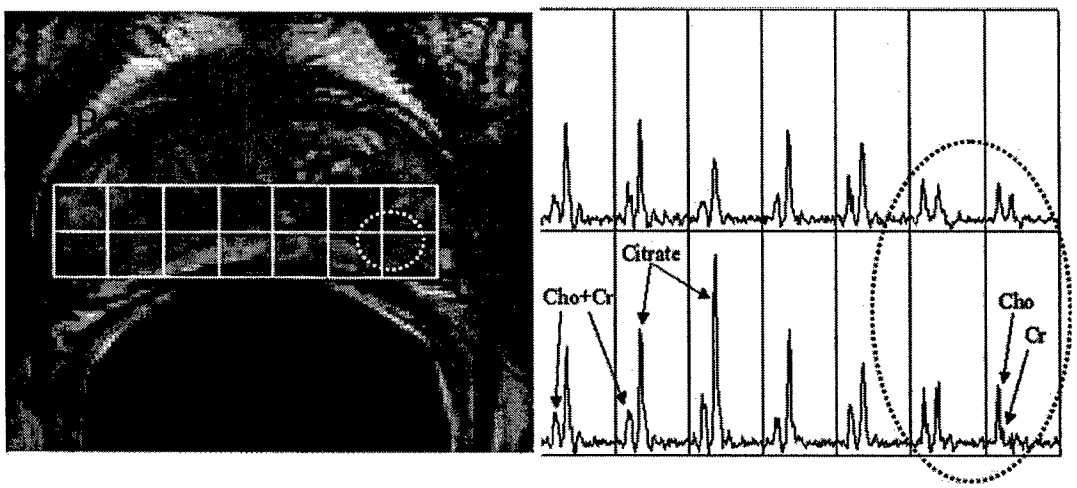

Fig. 3. In situ magnetic resonance spectroscopy imaging of prostate gland. The MRI fails to detect a malignant locus outlined by the dotted circle. MRS detects the peripheral zone tumor site by the loss of the citrate signal. Taken from Kurhanewicz *et al.* [20].

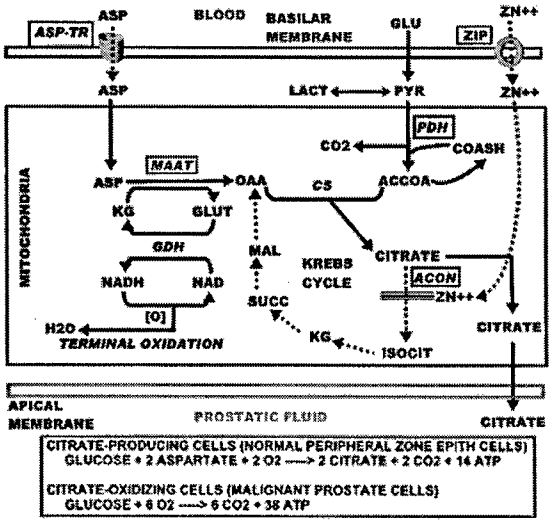

Fig. 4. The role of zinc in the metabolic pathway of net citrate production in prostate cells.

of zinc. The decreased zinc levels associated with the malignant cells results in increased citrate oxidation and decreased citrate levels. Therefore, the *in situ* citrate changes in normal versus malignant peripheral zone identified by MRS also represent the changes in zinc. Then it is further evident that the decrease in zinc and in citrate occurs in the early development of malignancy and persists through advance stage malignancy in the peripheral zone.

The combination of the consistent results of the direct assay of zinc described in the previous section with the consistent results of the citrate changes that must be preceded by zinc changes, and supplemented with additional information described below, provide the compelling conclusion that the lost ability of malignant cells to accumulate zinc plays an important and indispensable role in the pathogenesis of PCa. It is important to point out that the studies and the relationships that we have described apply to the organ-confined primary site (i.e. peripheral zone) malignancy. There is no existing information regarding the zinc/citrate relationships in the metastatic cells after the primary malignant cells intravasate and progress through invasion of and development in the distant metastatic tissue sites.

What is the cause of the decrease in zinc accumulation in the malignant cells?

A critical issue that awaits elucidation is the cause or mechanism responsible for the lost ability of the malignant cells to accumulate zinc. Although they share essentially the same interstitial fluid environment as other mammalian cells, the normal peripheral zone epithelial cells accumulate cellular levels of zinc that are several-fold higher than most other mammalian cells. The zinc level within cells is the net result of zinc uptake by the cells and zinc export out of the cells. The initial source of cellular zinc is its uptake from interstitial fluid, i.e. derived from blood plasma. Once within the cell, the zinc is either retained or exported out of the cell.

Recent studies [30, 31] have shown that the zinc uptake transporter, ZIP1, is important in the uptake and accumulation of zinc by prostate cells. Up-regulation of ZIP1 in prostate cells increases zinc accumulation, which inhibits cell growth and increases net citrate production. Correspondingly, down-regulation of ZIP1 decreases zinc accumulation in prostate cells. These studies have established ZIP1 as an important transporter that is responsible for the accumulation of zinc in prostate cells. Rishi *et al.* [32] reported that ZIP1 (and ZIP2) expression in peripheral zone glandular epithelium of black males is down-regulated as compared to its expression in white males, which coincides with the race-associated higher incidence of PCa in African-Americans. These relationships suggested the possibility that the decrease in zinc in malignant prostate glands (as shown in Figure 2) might be due to the down-regulation of ZIP1 expression. Our recent studies in collaboration with Roswell Park Cancer Institute (Dr. Keshav Singh) and with Claflin University (Dr. Omar Bagasra) show that ZIP1 gene expression, which is expressed in normal peripheral zone glandular epithelium, is down-regulated in adenocarcinomatous glands (manuscript in preparation; see Figure 2 for representative data). Correspondingly, the zinc level is depleted in the malignant glands. Consequently, the down-regulation of ZIP1 transporter expression appears to be an essential factor in the development and progression of malignant prostate cells. Since ZIP1 is expressed in the malignant prostate cell lines, its down-regulation in malignant glands *in situ* is likely due to gene silencing by *in situ* conditions that are not represented under *in vitro* conditions of the malignant cell lines. Beck *et al.* [33] reported that RT-PCR determination of ZIP1 expression revealed no significant difference between resected normal and cancerous prostate tissue. Unlike the *in situ* study of Rishi *et al.* and our unpublished studies, Beck *et al.* used extracted RNA from relatively large tissue samples that likely contained non-malignant tissue that might mask any differences due to malignant cells.

An increase in export of zinc could also decrease zinc accumulation by "true" malignant cells. However, no information currently exists concerning the functional role of zinc exporters in prostate cells. Beck *et al.* [33]

reported that ZnT-4 was decreased in peripheral zone malignant tissue tissue when compared to normal peripheral zone tissue samples. ZnT-4 is associated with the sequestering of cytosolic zinc into organelles, and not involved as a plasma membrane zinc exporter. Moreover, a decrease in Zn-T4 would not be associated with a decrease in cellular zinc level, even as a secretory process. In contrast, Zn-T1 expression was unchanged in malignant versus normal peripheral zone. Zn-T1 does function as a plasma membrane-associated zinc exporter in some cells and presumably in prostate cells. Hasumi *et al.* [34] reported that Zn-T1 expression was significantly lower in malignant prostate tissue samples when compared to BPH samples, which led them to conclude that Zn-T1 was not likely to be associated with the decreased zinc accumulation in "true" malignant cells. Consequently, a possible role of altered expression of zinc exporters in the genetic/metabolic transformation of the malignant cells *in situ* is not evident, but more research is required regarding this issue.

Zinc and the metabolic concept of prostate malignancy

The relationships that we have discussed lead to the concept of the role of zinc in the pathogenesis of PCa as represented in Figure 5. In this concept two transformations are essential for the development of a full-potential malignant cell. The normal epithelial cell expresses ZIP1 and is a zinc-accumulating citrate-producing cell. The malignant process is initiated by a genetic transformation of the normal epithelial cell to a neoplastic cell. The neoplastic cell, for reasons unknown at this time, becomes susceptible to conditions that cause the down-regulation of ZIP1 gene expression. The cell enters a pre-malignant stage in which the ability to accumulate zinc is lost. As the cellular zinc level is decreased, the inhibitory effect of zinc on m-aconitase and citrate oxidation is removed. This metabolic transformation results in increased citrate oxidation, and the Krebs cycle becomes fully operational. The cell becomes a malignant cell in which the energetic and biosynthetic requirements for the manifestation of the malignant activities are fulfilled.

There are two established consequences of this metabolic transformation. The bioenergetic consequence of net citrate production (which occurs in the normal epithelial cell) is energetically costly since the cells derive only 37% of the ATP that is generated by the complete oxidation of citrate (Figure 3). The metabolic transformation to an energy-efficient citrate-oxidizing malignant cell provides the increased energetic requirement for the malignant process. Another consequence of the decrease in zinc levels relates to the growth effects of zinc. Exposure of prostate cells to zinc induces mitochondrial apoptogenesis [35–37]. Therefore, the decrease in zinc accumulation in the malignant cells eliminates its apoptotic effect, thereby permitting the proliferation of the malignant cells. It is also important to note that the metabolic/bioenergetic transformation of normal prostate epithelial cells to malignant cells is unique when compared to the metabolic/bioenergetic relationship in most other tumor cells ([38–41] for reviews). The highly specialized normal citrate-producing prostate epithelial cells, of necessity, have an impaired citrate oxidation and truncated Krebs Cycle. They exhibit a low respiration and high aerobic glycolysis, which is in contrast to most other normal mammalian cells. The lost ability of the malignant prostate cells to accumulate zinc results in the metabolic transformation of the energy-inefficient citrate-producing sane cell to an energy-efficient citrate-oxidizing malignant cell. In contrast, most tumor cells are derived from normal cells that exhibit the typical

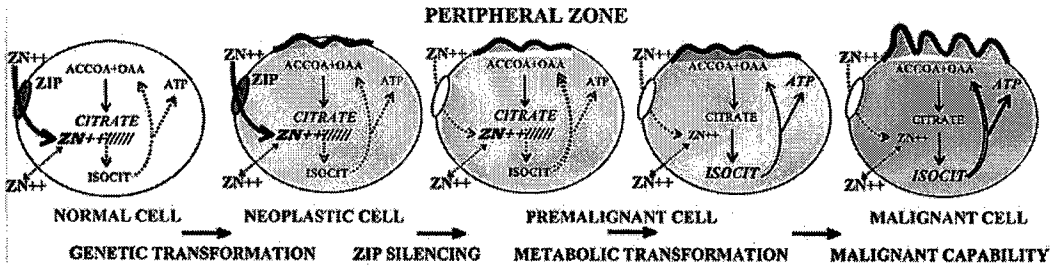

Fig. 5. Concept of the role of zinc in the pathogenesis of PCa. The normal cell possesses a zinc-accumulating mechanism (e.g. ZIP transporter), which results in high cellular zinc accumulation, inhibition of m-aconitase and citrate oxidation, and citrate accumulation. The genetic transformation provides the malignant potential of the neoplastic cell. The neoplastic cell loses the ability to accumulate zinc (e.g. the down regulation of ZIP1 expression) and becomes a dormant premalignant cell. As the cellular zinc levels decline, the premalignant cell undergoes a metabolic transformation in which the inhibition of m-aconitase is eliminated so that citrate oxidation via the Krebs cycle proceeds with the generation of ATP. The resulting malignant cell is now metabolically and bioenergetically capable of performing its malignant activities. In addition, the depletion of zinc eliminates its apoptotic effect and allows the proliferation of the malignant cell.

aerobic metabolism with the complete oxidation of glucose via a functional Krebs cycle, and are energy-efficient citrate-oxidizing cells. The tumor cells generally exhibit a high aerobic glycolysis, do not oxidize citrate, and are energy-inefficient. Relative to these metabolic relationships, the metabolism of prostate malignancy is opposite to the metabolism of other tumor cells. This emphasizes that generalized metabolic/bioenergetic relationships of most mammalian cells cannot be presumed to be representative of or applicable to prostate cells.

Does zinc provide a rational approach to the prevention and treatment of PCa?

This is a critical issue that currently confronts the scientific/medical community. In the following description, we will attempt to provide the essential information that supports the concept of the efficacy of zinc against PCa versus the information that contradicts this concept.

The rationale in support of this concept resides first on the scientific credibility and consistency of the clinical relationships presented in the preceding sections that (1) the malignant prostate cells *in situ* virtually never exist as zinc-accumulating cells, and (2) the metabolic transformation to neoplastic cells that have lost the ability to accumulate zinc is essential for the manifestation of their malignant activities. Experimental studies of other reported effects of zinc provide additional support. Uzzo *et al.* [42] reported that physiological levels of zinc inhibit NF-0B activity in androgen-independent PC-3 and DU-145 PCa cells, reduce constitutive expression of the anti-apoptotic protein c-IAP2, and sensitize malignant cells to apoptosis induced by cytotoxic agents. Ishii *et al.* [43] reported that the ability of LNCaP cells to invade Matrigel was strongly suppressed by Zn^{++}. These results suggest that zinc might suppress the invasion and metastasis of the malignant cells in PCa. However, the high zinc concentration employed [43] raises question of the physiological relevancy of these *in vitro* effects. In another study Ishii *et al.* [44] found that aminopeptidase N purified from human prostate was irreversibly inhibited by low concentrations of zinc ($Ki = 11.2$ μM); which could be associated with invasive capability. *In vitro* studies have revealed that exposure of malignant prostate cells to physiological levels of zinc results in apoptosis [35, 36]; and that this effect of zinc also occurs *in vivo* in PC-3 xenograft tumors [37]. The combination of the zinc relationships that exist *in situ* in PCa coupled with *in vitro* effects of zinc provides a compelling and plausible basis to propose that creation of conditions that restore the accumulation of zinc in the malignant cell (for treatment) or in the premalignant cell (for prevention) will be efficacious against PCa.

In contrast, some reports suggest that zinc might be a contributing factor associated with an increased risk of PCa. Therefore, a critical assessment of those reported adverse effects is essential. The reported effect of zinc enhancement of telomerase activity [45] coupled with the report of increased telomerase association with malignant prostate tissue [46] is cited as a possible mechanism for zinc facilitation of progression of advanced stage PCa. The study involved the *in vitro* exposure of DU-145 cells to 30 and 100 μM Zn^{++}; i.e. Zn^{++} levels that would never exist *in vivo*. Zinc (50 μM) reportedly antagonizes biphosphonates [47], which are thought to be protective against bone invasion. This was an *in vitro* study with a PC-3 cell line, and no data or experimental description was presented relating to the zinc effect so that an analysis of the effect is not possible. However, the use of 50 μM zinc, presumably as Zn^{++}, would be most unphysiologic and non-existent in cells. The cytosolic level of Zn^{++} is negligible; being in the nM-fM range. Samman *et al.* [48] reported that ingestion of high zinc levels by subjects impaired antioxidant defense that could be associated with cancer development. However, the report found this effect in females, but there was no such effect observed in males. The reported adverse effect of excessive zinc levels (150 mg of elemental zinc twice a day for six weeks) on the immune system [49] might be contributing factor in the progression of PCa. This dose is more than twice the mean zinc level that was reported [10] to increase the risk of advanced stage PCa. If a compromised immune system is a contributing factor, one would expect other serious immunological conditions, especially given the long duration required for PCa to develop and progress. Moreover, recent studies [50–53] demonstrate the importance of supplemental zinc for immune response, especially in the elderly who normally develop lowered bioavailabilty of zinc. The reported zinc-induced increase in circulating testosterone [54] has been considered as a potential factor that promotes PCa based on the supposition that testosterone is directly related to prostate carcinogenesis. Such a relationship has not been established, and several studies (e.g. [55–58]) show that the circulating testosterone level either is not implicated in PCa or that diminished circulating testosterone is associated with PCa. Moreover, the adverse effect of zinc reported by Leitzmann *et al.* [10] occurs in advanced cancer, which is androgen-independent; but not in early stage cancer, which is androgen-dependent. Furthermore, a positive correlation between elevated serum zinc levels and PCa has not been established. Reported studies indicate that plasma zinc levels are unchanged [59] or lower [60–63] in

PCa subjects, but the plasma levels in subjects prior to the diagnosis of PCa is not known. Consequently, in contrast to the strong clinical and experimental evidence for a preventative effect of zinc against PCa, Leitzmann *et al.* [10] appropriately acknowledge "Strong evidence to support a specific mechanism for this [increased risk of advanced prostate cancer] association is lacking at present." This does not dismiss such a possibility, which, if exists, must await confirmation by further investigation.

Evidence that zinc treatment is efficacious against prostate tumor growth

There had been no reported experimental studies or controlled clinical trials of the efficacy of zinc treatment against PCa. We recently employed the PC-3 xenograft model in nude mice for an initial preliminary study of the possible anti-tumor effects of zinc treatment [37]. A subcutaneous sustained dose equivalent to an adult human daily dose of approximately 50–100 mg zinc was employed. Figure 6 shows that this treatment regimen significantly increased the plasma zinc level. The zinc level of host prostate tissue was also markedly increased, as was the citrate level. This confirms that the accumulation of zinc inhibited m-aconitase and citrate oxidation. Zinc treatment significantly decreased prostate tumor growth. The tumor cells from the treated animals exhibited increased zinc accumulation, increased citrate level, and increased apoptosis. These *in situ* effects are consistent with the actions of zinc that we described in the previous sections, and are consistent with the concept of zinc treatment being efficacious against prostate tumor growth. The critical issue is whether or not such results will be exhibited in human PCa. The results obtained in the PC-3 cell tumorigenic model supports the concept that conditions that will increase the cellular accumulation of zinc will abort/arrest prostate malignancy.

An important issue is, "How to induce an increase in the uptake and accumulation of zinc in the pre-malignant cells (for prevention) and in the malignant cells (for treatment); which, in either case, the zinc uptake transport process is deficient (e.g. ZIP1 down regulation). Are there agents or conditions that will facilitate the zinc transport process in these cells?" It has been demonstrated that the uptake and accumulation of zinc by prostate cells is regulated by prolactin and testosterone ([64], for review [4]), as is the expression of ZIP1 [30]. Consequently, it is plausible to propose that hormonal treatment in combination with dietary zinc supplement could provide an effective anti-tumor regimen. In practice, the use of prolactin or testosterone treatment would likely have serious contraindications. However, their effects on zinc accumulation and ZIP1 expression provide a rationale for the discovery and development of other agents that would enhance zinc uptake by malignant prostate cells. Genetic manipulation such as up-regulation of ZIP1 to increase zinc accumulation and down-regulation of m-aconitase to inhibit citrate oxidation, or the use of selective m-aconitase inhibitors (e.g. such as a prostate-specific fluoroacetate analog), all provide potential anti-tumor approaches based on the zinc/citrate relationship.

One of the important challenges is the identification of appropriate tumorigenic models that exhibit the zinc/citrate metabolism transformation that characterizes the malignant activities of human PCa. Extensive and numerous reports exist of prostate tumor models

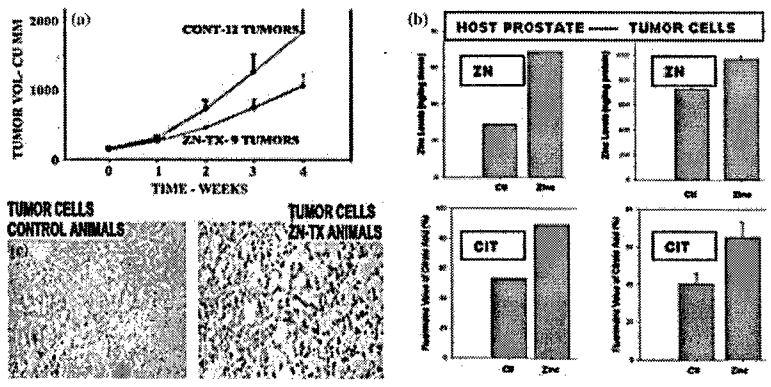

Fig. 6. Anti-tumor effects of zinc treatment against prostate cell tumorigenicity in nude mice. The PC-3 cell xenograft tumorigenic was employed. Animals were administered zinc via subcutaneous implanted sustained-release minipumps containing zinc chloride. (a) Effect on tumor growth. (b) Effect on zinc and citrate accumulation in the host prostate tissue and in the tumor cells. (c) Effect on tumor cell apoptosis (Tunel Assay).

purporting to be representative of PCa. These include xenograft tumorigenic models with human malignant cell lines, and spontaneous or induced prostate tumors in experimental animals. However, the important issue is whether or not the prostate tumor models exhibit the metabolic characteristics of the "true" *in situ* malignancy that occurs in PCa. Unfortunately, virtually no studies exist to determine if these models exhibit the zinc/citrate metabolic transformation that is an essential requirement in "true" malignancy in PCa. The PC-3 xenograft model that we employed in the aforementioned preliminary study is promising, but some issues need to be resolved. That the PC-3 tumor cells will accumulate zinc derived from circulation and exhibit the expected zinc-indiced effects is evident. However, these cells in culture express ZIP1, and this would not be representative of the lost expression of ZIP1 that occurs in the malignant cells *in situ* in human PCa.

We must also emphasize that the zinc/citrate relationships that we have described and have represented in Figure 4 are applicable to organ-confined PCa. There is virtually no clinical information regarding zinc and citrate-related metabolism of the metastatic cells that reside in the distant tissue sites. The available malignant prostate cell lines have been derived from metastatic lesions, but it is not confirmed or evident that these cells reflect the *in situ* characteristics of their progenitor metastatic cells. These issues exemplify the need to develop appropriate human PCa models, and to define carefully the characteristics and limitations of such a model. This is an important area of concentrated research and development that is essential to study the mechanisms of zinc/metabolic relationships in the pathogenesis of prostate malignancy and the efficacy and mechanisms of zinc in the treatment and prevention of PCa.

A basis for efficacious use of dietary supplemental zinc against PCa?

An important issue is whether or not the use of dietary supplemental zinc would be efficacious against the development/progression of PCa. This issue involves a number of considerations. The ability of prostate cells to accumulate zinc is dependent upon two factors: the availability of exogenous zinc for import into the cells; and the activity of transporter(s) required for zinc accumulation. Present information identifies ZIP1 as a key transporter for zinc accumulation; but, future research could reveal the additional involvement of other zinc transporters. It is important to recognize the relationship of plasma zinc, interstitial fluid zinc, and zinc uptake transport by ZIP1 or any zinc uptake transporter. The availability of exogenous zinc for uptake is dependent on the interstitial fluid level of transportable (mobile) zinc. The normal concentration of plasma zinc approximates 15 μM; of which ~70% is protein bound and the remaining ~30% is bound to low molecular weight ligands. There is essentially no free Zn^{++} ion pool in plasma. The interstitial fluid being an ultrafiltrate of plasma contains the low molecular-bound zinc, i.e. ~4.5 μM zinc. Another important factor is the binding affinity of zinc with its ligands. For zinc to be transportable requires that it not be "tightly" bound to its ligand, which appears to be a binding affinity of log K_f~10 or lower. The cellular uptake of zinc is dependent upon the K_m of the transporter; which, for ZIP1 is ~3 μM zinc [31]. At normal plasma zinc levels, the concentration of mobile reactive zinc (transportable zinc) approximates the K_m value of the transporter. Therefore, the prostate cells are capable of effectively importing and accumulating zinc. If the plasma level of zinc decreases, the availability of transportable zinc in the interstitial fluid will be disproportionately decreased. This is due to the fact that the ligands that tightly bind zinc exert preference for the available zinc. This immobile pool of zinc will not be affected unless the total concentration of zinc decreases below the binding concentration of these ligands. Therefore, the decrease in total plasma zinc levels will be reflected totally as a decrease in the concentration of transportable zinc. As this concentration of interstitial fluid transportable zinc decreases below the K_m value of the transporter, the effectiveness of the transporter decreases and the uptake and accumulation of zinc decreases.

Plasma zinc and tissue zinc levels generally declines with aging [65–69]. Decreased plasma zinc level is often associated with PCa [60–63]. Zinc uptake transport activity (ZIP1) is diminished in malignant prostate cells. The co-existence of these conditions provides the optimal situation for the manifestation of overt clinical malignancy. Of these, the availability of plasma zinc can be altered.

The use of dietary supplemental zinc is important to achieve and to maintain a sufficient level of interstitial fluid zinc. In the absence of zinc supplement in which a zinc deficiency is prevalent in the elderly male population, cellular zinc uptake will be depressed even if the cell expresses ZIP1. If the concept of malignancy occurs as represented in Figure 5, overt malignancy will not be evident until a sufficient population of malignant cells develops. This requires the transition of the neoplastic cell that is not yet capable of malignant activity to the malignant cell. It is important to note that the down regulation of ZIP1 is an epigenetic effect, and is not a

spontaneous chromosomal deletion or fatal mutation effect. In addition, the down regulation of ZIP1 expression does not immediately remove the existing ZIP1 transporter so that the decline in transporter activity and in the cellular accumulation of zinc is progressive not spontaneous and becomes more pronounced in the succeeding generations of the malignant cells. The slow development of a significant population of neoplastic cells with down-regulated ZIP1 would be a contributing factor in the slow-developing slow-growing characteristic of prostate malignancy. Thus, in the presence of normal plasma zinc levels, the transition period to malignancy is prolonged. Under conditions of a decrease in plasma zinc, as occurs in the elderly male population, cellular zinc uptake will be depressed even in the cell neoplastic/premalignant cells that exhibit ZIP1 transporter. This will eliminate the requirement for the down-regulation of ZIP1 to prevent the accumulation of cellular zinc. The metabolic transition of the neoplastic cell to the malignant stage will be accelerated. In this circumstance, the use of zinc supplement to alleviate a low plasma zinc status would be efficacious against the development of overt clinical malignancy. This might also explain the reported observations of low plasma zinc association with PCa.

Another consideration is the effect of elevated plasma zinc levels (interstitial fluid level of zinc) on the malignant prostate cells that exhibit the loss of operational zinc uptake transport capability (down-regulation of ZIP1). It is possible that a significant increase in transportable zinc will result in zinc uptake by these cells. This could result if a marked decrease, but not complete loss, of ZIP1 occurs in some population of the malignant cells as discussed above. Also, in the absence of specific zinc uptake transporters (e.g. ZIP1), other zinc-associated transport processes might be functional under conditions of high zinc availability that would not occur under normal or low plasma zinc levels. Cellular zinc uptake by simple diffusion of the transportable zinc-ligands is minimal at normal concentrations of circulating zinc. However, this could be increased by an elevated concentration of the transportable zinc-ligands. The use of dietary zinc supplement that can sufficiently increase plasma zinc levels to achieve these effects would be efficacious against PCa. These possible effects of zinc supplement need to be investigated.

What have epidemiological studies revealed?

A number of epidemiological studies and reviews that involved or examined the efficacy of dietary zinc against PCa have been reported [5–10, 70–72]. Some reports [5–7] indicated that dietary zinc had no apparent beneficial or harmful effect on PCa risk. Kristal *et al.* [8] reported a significant 45% reduction in the risk of PCa by zinc supplement; and Key *et al.* [9] also concluded that zinc might be beneficial against PCa. In contrast, Leitzmann *et al.* [10] reported a significant increase in the risk of advanced stage PCa by excessive zinc supplement. Kolonel *et al.* [11] also reported that supplemental zinc might be associated with PCa in men 70 years and older, but not in men under 70 years old. In a subsequent review [70], Kolonel *et al.* concluded, "The data on other dietary components [including zinc] that have been examined with regard to prostate cancer etiology ... are too incomplete at this time to draw any inferences as to their importance."

One should expect that any study that reports a statistically significant outcome leading to a definitive conclusion should be observable in most other past or future comparable studies with proper statistical analysis. Yet, no consistent observation has resulted from the epidemiologic studies. The divergent results and absence of unanimity regarding the effects of dietary zinc on PCa raise many issues and questions. Why, in the face of the compelling clinical and experimental evidence described in the previous discussions, is there not a definitive and consistent protective effect of dietary zinc supplement against PCa revealed by these epidemiologic studies? Why does Kristal *et al.* [8] report a significant dose–response protective effect for zinc, and no such effect is reported by other [5–7, 10] epidemiologic reports? Why does Leitzmann *et al.* report a significant increased risk; and Kristal *et al.* observe no such risk? Are we dealing with issues of zinc dosage; population differences; population selection bias; application of statistical procedures; other factors? Among all the epidemiologic studies, the study of Kristal *et al.* [8] that reports a preventative effect of zinc supplement, and the study of Leitzmann *et al.* [10] that reports a harmful effect of zinc supplement are, arguably, the most comprehensive studies. An important distinction of the Leitzmann *et al.* study from other epidemiologic reports is the inclusion of high zinc supplement use (>100 mg/day) and the extended duration (>10 years), i.e. the conditions that purportedly impose an increased risk of advanced PCa. However, at lower levels of zinc supplement, no effect on early or advanced PCa is reported. Seemingly, this is contrast to and inconsistent with the beneficial effect of the regular use of zinc supplement observed by Kristal *et al.* [8] and suggested by Key [9], but other reports [5–7], 70] also failed to observe this protective effect. The Kristal *et al.* report focused on the weekly frequency association of zinc supplement use, but provided no information regarding the dose of zinc supplement.

Subsequent to their initial study, Leitzmann and Giovannucci [73] provide data that show some possible protective effects of zinc as exemplified by a 41% decrease in organ-confined cancer and ~24–29% decrease in cancer in other zinc-user groups; which would be similar to the protective effect reported by Kristal *et al.* [8]. The discrepancies associated with the epidemiologic studies need to be resolved. The complex multi-variate factors that are associated with the use of supplemental dietary zinc and their impact on PCa need to be understood and considered.

Gastro-intestinal factors

The complexity of interacting multiple dietary factors that affect the intestinal absorption and assimilation of zinc [74–76] is a likely major factor in these diverse results. For example, the level of phytate and calcium, iron, and numerous other ingested nutrients, and the chelate form of zinc supplement can alter the absorption of zinc. An important factor that has not been considered in these epidemiological studies is the effect of the level of zinc ingestion on the circulating level of zinc. The intestinal absorption of zinc, along with the gastrointestinal secretion and excretion of zinc, provides a homeostatic mechanism that regulates the systemic level of zinc. For example, a low intestinal zinc level up-regulates zinc absorption, and excessive intestinal zinc level down-regulates zinc absorption. The supposition of most of the epidemiological studies has been that the amount of ingested zinc is approximated by the amount of zinc that is absorbed and assimilated. When all these gastrointestinal interacting factors are considered, this supposition becomes untenable. Any direct effect of zinc on normal or malignant prostate cells is dependent on the circulating level of zinc and its uptake by the cells; not on the intestinal level of zinc. In lieu of the difficulty in the determination of prostate zinc levels, the effect of the ingested level of zinc on the systemic level of zinc is important to establish; and that information is lacking in these epidemiological studies. As already noted, reports have demonstrated that plasma zinc levels were decreased [60–63] or unchanged [59]; but not increased in PCa subjects.

Calcium supplement effects

It is interesting to note that the adverse effects of high zinc supplement resemble the effects of calcium. Several studies [77–81] have shown that increased daily intake of dietary calcium increases the incidence of advanced PCa. In the report of Leitzmann *et al.* [10] the level of calcium intake (about 1919 mg/day) in the high zinc-user group was essentially identical to the level of calcium intake (@2000 mg/day) that other reports have shown to be associated with advanced PCa. They make the important observation that residual confounding by supplemental calcium intake with zinc supplement use cannot be ruled out. Their report raises the need to determine the interesting possibility of a mechanism of calcium and zinc inter-relationships in PCa.

Potential carcinogenic effects of contaminants

Another important consideration is the possible effect of contaminants that exist in commercial zinc supplement products. Krone and Harms [82] suggested that the presence of cadmium, which has been implicated in the etiology of PCa, in zinc supplements could be a contributing factor in the increased risk of advanced PCa long-term high-zinc supplement use. We would add that an additional possible contributor is the presence of lead contamination that exists in zinc chelate products. Interestingly, a positive correlation has been shown [83] among an increase in plasma lead level, a decrease in plasma zinc level, and subjects with PCa. The report concludes that exposure to lead results in PCa.

A combined increase in cadmium and lead consumption that results in their systemic accumulation would likely result from the prolonged use of supplemental zinc products; and this could be an important inducing factor in PCa. Such a possibility is increased by the observation [10], "By contrast, zinc obtained from food sources [compared to high zinc supplement] was not associated with prostate cancer risk (data not shown)", which also reflects the observation of Kolonel *et al.* [11]. This observation would also implicate contaminating components of commercially available zinc supplement as more likely than zinc to be associated with the risk factor. This possibility is amplified by the observation that extended daily use of low- or moderate-dose zinc supplement for >10 years has the same adverse effect as the short-term use of high-dose zinc supplement. Contaminants such as cadmium and lead will accumulate by chronic exposure to low doses and/or by shorter term exposure to high doses of such contaminants, which is not the case for zinc exposure, which is homeostatically and physiologically regulated. In addition, the data presented by Leitzmann and Giovannucci [73] suggest that long-term use of moderate dose of zinc supplement will have the same adverse effect as long-term use of high zinc supplement in promoting advanced stage PCa. No evidence exists that chronic use of low-moderate levels of dietary zinc will result in zinc accumulation in tissues over time, especially as compared to the chronic use of high levels of zinc

supplements. However, the availability and accumulation of potentially deleterious contaminants such as lead and cadmium would increase in proportion to the level of zinc supplement consumption.

The likelihood of a role of cadmium and lead contaminants in increased risk of high zinc supplement use seems even more likely in view of the recent observation of the importance of ZIP1 down regulation in prostate malignancy. There are no natural biological transporters for non-biologic agents such as cadmium and lead. Their uptake and accumulation by cells is achieved by their competition with the natural substrates of biological transporters. ZIP1 is a candidate transporter for cadmium and lead. Cadmium at high concentrations inhibits zinc uptake transporter in prostate cells [84]. No information exists regarding lead, but it is reasonable to expect it to inhibit zinc transport by ZIP1. A possible mechanism for the carcinogenic effect of cadmium and lead could involve their inhibition of zinc uptake and accumulation by the neoplastic prostate cells thereby facilitating the depletion of zinc and enhancing the malignant process.

What interpretations can be drawn from the epidemiologic studies?

The divergent and inconsistent results and conclusions of the epidemiologic studies regarding the beneficial or harmful effects of supplemental zinc usage on PCa is apparent. However, some consensus and important implications can arise from this confusion.

(a) A clear distinction must be made between "effects of zinc" and "effects of zinc supplement". The former should imply that zinc is the cause of an effect; the latter implies the zinc supplement results in an effect that might or might not be due to zinc.
(b) A distinction must be made between an effect of zinc or zinc supplement on the prostate, or a systemic effect that impacts on the prostate.
(c) A consensus derived from essentially all the epidemiologic studies is that the use of supplemental zinc at levels less than ~75 mg/day) does not exhibit any increased risk factor for PCa. Some evidence indicates a protective effect of zinc supplement at moderate levels. The recommended dietary allowance is 11–15 mg a day for men, but somewhat higher levels might be advisable for elderly males.
(d) The chronic use of excessive levels of zinc supplement might increase the risk of advanced PCa and should be avoided until additional information becomes available.
(e) The potential adverse effects of excessive levels of zinc supplement likely involves the effects of multivariate factors separate from or in concert with zinc. The possible effects of contaminants (notably cadmium and lead) in commercial zinc supplement products as contributors to increased risk of PCa need to be investigated.
(f) The consensus of all the epidemiologic reports is that further studies are required to resolve the issue of the efficacy of zinc against PCa.

Thus, while generally inconsistent and confusing, the collective epidemiologic studies provide some important information and insights, and provide a road map for the future studies.

Summary and conclusions. What is the current state regarding zinc and prostate cancer?

A. The pathogenesis of PCa involves the lost ability of the malignant cells to accumulate zinc as an essential step in the malignant process. High cellular zinc accumulation is detrimental to the malignant activities of malignant prostate cells.
B. Compelling clinical and experimental evidence supports the expectation that an appropriate regimen of zinc supplement that would increase the uptake of zinc by malignant prostate cells should be efficacious against the development and progression of PCa.
C. Due to lifestyle, eating and dietary habits, and physiological effects of aging, the elderly male population is normally predisposed to conditions of zinc deficiency, which can increase their susceptibility to PCa and other pathologies. Therefore, appropriate zinc supplement use is important to the health of this male population.
D. Evidence exists that components and factors that are associated with the excessive use of zinc supplements might contribute to an increased risk of advanced PCa. Potentially important in the chronic use of high zinc supplements is the possibility that contaminants of zinc supplement products such as cadmium and lead, not zinc itself, contribute to an increased risk of PCa.
E. The inconsistent and inconclusive results of the various epidemiologic studies lead to the conclusion that the effect of dietary and supplemental intake of zinc on PCa is complex and confounded by unknown multivariate factors. An understanding of the confounding conditions that could be associated with zinc supplement use is essential to the analysis of epidemiological reports of the effects of zinc on PCa.
F. Public pronouncements regarding any effects (beneficial or detrimental) of supplemental zinc usage

and PCa should be judicious and balanced. The tendency to warn the public of the use of excessive zinc supplement and increased risk of PCa can have unintended consequences. Such pronouncements are likely to be interpreted by the public as a reason to suspend or avoid any use of zinc supplement. This could have deleterious effects on the elderly male population that is predisposed to zinc deficiency and its attending potential health consequences, including PCa.

G. All the epidemiological reports reach the consistent conclusion that the role of zinc in the pathogenesis and progression of PCa and its efficacy, or lack thereof, in the treatment and/or prevention of PCa are extremely important issues that warrant further investigation. Well-controlled experimental and clinical studies of the efficacy of zinc alone and in combination with other supplements are required, along with basic research studies of the mechanisms of zinc effects on normal and malignant prostate. Any future epidemiologic studies regarding supplemental zinc and PCa must be planned and controlled with an understanding of the multivariate conditions and factors that will affect the bioavailability and potential effects of zinc on the development and progression of prostate malignancy.

H. The need for the development of experimental models that reflect the zinc/metabolic relationships that exist in human PCa is critical to an understanding of the role of zinc in the pathogenesis and progression of prostate malignancy, and in the assessment of zinc and interacting variables in the treatment and prevention of PCa and their mechanisms of action.

Acknowledgement

Studies of LCC, RBF, P. Feng were supported by NIH grants CA 79903, CA 71207, DK42839 and CA93443, and by DOD Grant DAMD 17-01-0072. Studies of OB were supported by DOD grant DAMD 17-02-1-0233.

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Down Regulation of Expression of hZIP1 Zinc Uptake Transporter and Depletion of Zinc in Prostate Cancer. A Possible Tumor Suppressor Gene

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ABSTRACT

Background: The genetic and molecular mechanisms responsible for and associated with the development and progression of prostate malignancy are largely unidentified. The peripheral zone is the major region of the human prostate gland where malignancy develops. The normal peripheral zone glandular epithelium has the unique function of accumulating high levels of zinc. In contrast, the ability to accumulate zinc is lost in the malignant cells. Compelling evidence exists that the lost ability of the neoplastic epithelial cells to accumulate zinc is essential in the development of malignancy. Recent studies identified ZIP1 as an important zinc transporter involved in zinc accumulation in prostate cells. Therefore, we investigated the possibility that down-regulation of *hZIP1* gene expression might be involved in the inability of the malignant cells to accumulate zinc. To address this issue, the expression of *hZIP1* and the depletion of zinc in malignant versus non-malignant glands of prostate cancer tissue sections were analyzed. *hZIP1* expression was also determined in malignant prostate cell lines. **Results:** *hZIP1* gene expression, ZIP1 transporter protein, and cellular zinc were prominent in normal peripheral zone glandular epithelium and in benign hyperplastic glands (also zinc accumulating glands). In contrast, *hZIP1* gene expression and transporter protein were markedly down-regulated and zinc was depleted in adenocarcinomatous glands and in prostate intra-epithelial neoplastic foci (PIN). These changes occur early in malignancy and are sustained during its progression in the peripheral zone. h*ZIP1* is also expressed in the malignant cell lines LNCaP, PC-3, DU-145; and in the nonmalignant cell lines HPr-1 and BPH-1. **Conclusions:** The studies clearly establish that *hZIP1* gene expression is down regulated and zinc is depleted in adenocarcinomatous glands.

The fact that all the malignant cell lines express *hZIP1* indicates that the down-regulation in adenocarcinomatous glands is likely due to in situ gene silencing. These observations, coupled with the numerous and consistent reports of loss of zinc accumulation in malignant cells in prostate cancer, lead to the plausible proposal that *hZIP1* is a tumor suppressor gene in prostate cancer.

Key Words: prostate cancer; zinc; ZIP1 zinc transporter; citrate; ZIP1 gene expression

Background

Despite the extensive clinical and experimental studies over the recent decades, the pathogenesis of prostate cancer remains unknown. The genetic and molecular mechanisms responsible for and associated with the development of malignant prostate cells and their progression are largely unidentified [for reviews see 1,2]. The major site for the development of prostate malignancy is the peripheral zone, which comprises about 70% of the prostate gland. It is well established that the normal peripheral zone has the function of accumulating extremely high zinc levels that are 3-10-fold greater than found in other soft tissues (Table 1). This capability resides in the highly specialized glandular secretory epithelial cells of the peripheral zone, which we characterize as "zinc-accumulating" cells. In contrast, the malignant prostate cells that develop in the peripheral zone do not contain the high zinc levels that characterize the normal secretory epithelial cells. As represented in Table 2, repeated studies consistently show that the zinc levels of malignant prostate tissue are about 60-70% lower than the normal prostate

tissue. Measurements of pure malignant tissue in the absence of normal glandular epithelium would reveal even lower zinc levels that would approximate the levels found in other soft tissues. 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 [3] and Vartsky et al [4] further reveals the critically important relationship that, in individual analyses, malignant tissue never exhibits high zinc levels (figure 1). In addition, Habib [5] reported that the decrease in zinc occurs early in malignancy. These persistent results, and the additional corroborating evidence presented below, firmly establish that the unique zinc-accumulating capability of the normal peripheral zone secretory epithelial cells is lost in the neoplastic transformation to malignant cells; and that zinc-accumulating malignant cells do not exist in situ in prostate cancer. For extensive presentations of the relationships of zinc in normal prostate and prostate cancer, we refer the reader to our recent reviews [6-9].

The established clinical and experimental evidence provides the basis for our concept (Figure 2) that zinc accumulation prevents the malignant activities of the neoplastic prostate cell; and that impaired zinc accumulation is an essential requirement for the manifestation of prostate malignancy. If such is the case, one should expect that the zinc-accumulating process that characterizes the normal glandular epithelium is absent or defective in the malignant cells. Until recently, no information had been available regarding the mechanism(s) of zinc accumulation in prostate cells. Recent studies [10-12] have established that the zinc uptake transporter, ZIP1, is important in the uptake and accumulation of zinc by prostate cells. Up-regulation of ZIP1 in prostate cells increases zinc accumulation; and, correspondingly, down-regulation of ZIP1 decreases zinc accumulation in prostate cells. In addition, Rishi et al [13] reported that ZIP1 (and ZIP2) expression in peripheral zone glandular epithelium of black males is down regulated as

compared to its expression in white males; which coincides with the race-associated higher incidence of prostate cancer in African-Americans. These relationships suggested the possibility that the decrease in zinc in malignant prostate glands might be due to the down regulation of ZIP1 expression.

In this report we show, for the first time, the down regulation of h*ZIP*1 gene expression, the loss of ZIP1 transporter protein and the depletion of zinc that is evident in malignant prostate glands. The evidence presented supports the likelihood that silencing of *ZIP1* gene expression in the neoplastic prostate cell is an essential step in the development of prostate malignancy; and that *ZIP1* is a tumor suppressor gene in prostate cancer. The studies were conducted independently at three different institutions, which strengthen the validity of these corroborating results.

Results

The studies presented in this report were conducted at the University of Maryland (UMaryland study), the Roswell Park Cancer Institute (Roswell Park Study), and Claflin University (ClaflinU study). Therefore the results will be presented as provided by each separate and independent study, followed by the discussion of the evidence and supporting basis for the genetic/metabolic concept of the role of zinc in prostate malignancy.

The UMaryland Study (RBF, PF, BM, LCC)

Earlier studies [10-12] demonstrated that ZIP1 is expressed in vitro in malignant prostate cell lines (PC-3 and LNCaP cells); and that this zinc uptake transporter functions in the uptake

and cellular accumulation of zinc. This caused us to initiate preliminary studies to determine if *ZIP1* gene expression and/or the level of the transporter protein might be down-regulated in malignant prostate glands in comparison to the expression in normal prostate glandular epithelium. Paraffin mounted serial sections of human prostate tissue was used for ZIP1 immumohistochemistry staining. Hematoxylin and eosin staining was used for pathologic evaluation of normal glands and adencarcinomatous foci. Figure 3A reveals the membrane-associated immunohistochemical identification of ZIP1 in the normal peripheral zone glandular epithelium. In contrast, the malignant glands were essentially devoid of demonstrable membrane-associated ZIP1. It is also apparent that ZIP1 is confined to glandular epithelium and is not demonstrable in the stromal tissue. Figure 3B presents RT-PCR analysis of ZIP1 expression in tissue extracts of malignant tissue versus benign hyperplastic (BPH) glands; which, like normal peripheral zone, are zinc-accumulating glands. The results demonstrate a relatively high level of ZIP1 gene expression in BPH glandular tissue as compared with a barely detectable expression level in malignant tissue. These results provided the initial preliminary evidence that indicated that down regulation of ZIP1 expression is associated with malignant prostate tissue.

We previously reported the identification by Western blot of the presence of ZIP1 in PC-3 and LNCaP cells under standard culture conditions. These are malignant cell lines that were derived from metastatic prostate tissue. For correlation with the human tissue results, we proceeded to determine the presence of ZIP1 transporter in these cells by immunocytochemistry. Figure 3C shows the localization of ZIP1 in the plasma membrane; which is similar to the localization in normal peripheral zone glandular epithelium. The retention of this gene expression in LNCaP, PC-3, and DU145 (not shown) cells demonstrates that the absence of ZIP1 expression in the malignant glands in situ is not due to the deletion or fatal mutation of the gene.

No information exists regarding ZIP1 in metastatic cells in situ in prostate cancer. However, it seems most improbable that the gene would re-appear in metastasis, unless it was reversibly down-regulated in the primary site malignant glands. Therefore the results strongly implicate the epigenetic silencing of *hZIP1* gene expression in the primary site malignant cells under the in situ environmental conditions of the malignant prostate gland.

These initial observations dictated the importance of expanding the clinical investigation to establish conclusively that ZIP1 is down regulated in prostate malignancy and is associated with a decrease in the zinc accumulation in the malignant cells. To achieve this, independent studies were conducted at Roswell Park Cancer Institute and at Claflin University without prior knowledge of the results of the UMaryland study.

2. The Roswell Park Study (MMD, KKS)

The Roswell Park (RPCI) resources provided the opportunity to conduct ZIP1 immunohistochemical analysis of prostatic adenocarcinoma slides without identification related to patients. Twenty-two cases of prostatic adenocarcinoma were obtained from RPCI that contained both adenocarcinomatous foci and adjacent benign prostatic hyperplasia (table 3). Four of the cases contained normal prostatic glands and five cases contained prostatic intra epithelial neoplastic foci (PIN). The tumors were graded according to the World Health Organization grading system [14]. Grade 1 is defined by well differentiated glands with minimal anaplasia in which the nuclei are almost uniform with minimal variation in size and shape, and few detectable nucleoli. Grade 2 is defined by moderately differentiated glands with moderate nuclear anaplasia with many nucleoli. Grade 3 is defined by poorly differentiated or undifferentiated glands

showing marked anaplasia in which the nuclei showed marked variation in size and irregular shapes, vesicular, with marked abnormal mitotic figures.

Figure 4 shows the representative results of the ZIP1 immunohistochemical staining observed in normal peripheral zone glands, BPH glands, adenocarcinomatous glands and in PIN. The glandular epithelium of the normal glands and BPH glands (both being zinc-accumulating glands) exhibit immuno-positive ZIP1 staining. ZIP1 is localized predominantly at the basolateral membrane. In contrast, in the adenocarcinomatous glands and PIN, ZIP1 is negligible in the malignant cells so that the appearance of cell membranes is essentially absent. It is also evident that ZIP1 transporter is not detected in the stromal tissue, which corroborates the results of the U.Maryland study.

Table 3 is the summary of the immunohistochemical scoring of hZIP1 reactivity of tissue sections from 22 cases of prostate cancer. In this study, the analysis involves the comparison of ZIP1 in glands located in the same tissue section. This eliminates, or at least minimizes, any potential technical differences arising from antibody diffusion into the tissue sections and cells for immuno-reactivity. Any comparative differences observed in the immuno-reactivity in the different glands of the same tissue slice would be due to comparative differences in the level of hZIP1. Analysis of the 22 cases (figure 5) for the presence of glands that exhibit ZIP1 immuno-positivity results in a significant difference ($P<0.01$) between BPH glands (19 positive/3 negative) and adenocarcinomatous glands (7 positive/15 negative). Analysis for the presence of acini composed of >10% positive cells reveals that BPH glands exhibited this criterion in 50% (11/22) of the cases compared to 0/22 for the adenocarcinmatous glands (figure 6). The average scoring for the twenty-two cases (table 3) was also significantly lower ($P<0.01$) for the adenocarcinomatous glands (0.32) as compared to the BPH glands (1.68); i.e. ~5-fold difference.

Also, in every case in which the tissue sections showed a positive score for BPH glands, the adenocarcinomatous glands exhibited a lower score. Thus, all the criteria consistently reveal that the immuno-reactive ZIP1 is always reduced and mostly non-detectable in the malignant glandular epithelium. Another important observation is the absence of a correlation between the stage of prostate cancer and the down regulation of ZIP1. This reveals that the down regulation occurs early in the malignant process; which is consistent with the early changes in zinc levels.

As would be expected, the presence of normal peripheral zone glands in the malignant tissue sections is minimal, and insufficient for statistical analysis. However in three of the 4 cases, the normal glands exhibited the expected higher ZIP1 expression than the adenocarcinomatous glands, and gave results that were similar to BPH; both of which are zinc accumulating glands. In one case the normal gland was negative for ZIP1, which, seemingly, is an anomaly. However an important point needs to be considered. It is consistent with existing evidence (discussed below) that these metabolic changes occur before the appearance of histopathological evidence of malignant cells. Therefore, this "anomaly" might be due to changes that occur in a "premalignant" neoplastic condition that was histologically identified as "normal". Furthermore, in all five cases with PIN, the glands were either negative or + (none was ++ or +++), which mimics the profile of the adenocarcinomatous glands. It is striking that the combined PIN and adenocarcinoma glands showed no instance of ZIP1 positive cells that exceeded 10%. This could be supportive of a malignant relationship between PIN and adenocarcinoma; but further studies with additional PIN and normal peripheral zone glands are needed. Nevertheless, the Roswell Park study clearly establishes a consistent down-regulation of hZIP1 transporter in malignant prostate glands that corroborates and extends the results of the U.Maryland study, and is further corroborated by the following ClaflinU study.

The ClaflinU Study (AK-D, OB)

In this study ZIP1 mRNA expression (RT-in situ-PCR) and the relative level of zinc content were determined in the normal peripheral zone glands versus malignant glands from 38 prostate resections. The typical results represented in Figure 6 were consistently observed in all 38 prostate resections. The results show that *hZIP1* gene expression is evident uniformly in the epithelium of the normal peripheral zone glands. In contrast, *hZIP1* expression is markedly down regulated to the extent of not being demonstrable in the adenocarcinomatous glands. Of significance is the apparent down regulation of ZIP1 in early-stage as well as in advanced-stage malignant glands; which is consistent with the decrease in ZIP1 transporter protein shown in the Roswell Park study.

Correspondingly, Figure 6 shows the high level of cellular zinc that characterizes the normal glandular epithelial cells. In contrast, the stroma exhibits a low level of zinc. Therefore, the in situ zinc staining provides the expected differential in zinc between normal glandular epithelium and stroma. The marked reduction of cellular zinc in the epithelium of the adenocarcinomatous glands is apparent. Like the expression of ZIP1, the loss of zinc occurs early in malignancy. Due to the depletion of zinc in the malignant glands, the stromal zinc level gives the appearance of relatively higher zinc levels. Many studies have observed that zinc levels are greatly decreased in extracts of resected malignant tissue preparations (table 2). However, the ClaflinU study provides the first in situ detection of the depleted cellular zinc levels in adenocarcinomatous glands as compared to the high zinc levels in normal glandular epithelium. An important revelation is that the decrease in zinc level in the malignant glands is due to a decrease in the cellular accumulation of zinc. This establishes that the decrease in

intracellular zinc, and not impaired secretion of zinc into the lumen (prostatic fluid), is principally responsible for the decrease in malignant tissue zinc level. Thus, the results of the ClaflinU study are consistent with and corroborate the Roswell Park study and the preliminary results of the UMaryland study.

Discussion

The Zinc-Citrate Connection in Normal and Malignant Prostate

The results of the present study coupled with the numerous and consistent reports of others (table 2) [6-9 for reviews], provide direct overwhelming clinical and experimental evidence that, in prostate cancer, the lost ability of the malignant cells to accumulate zinc is a consistent event in the development of malignancy. However, the significance and further corroboration of this relationship requires the understanding and recognition of the unique role of zinc in normal prostate function and in prostate cancer. The major function of the human prostate gland peripheral zone (as in other animals) is the production and secretion of enormously high levels of citrate (table 1); which we refer to as "net citrate production" (figure 7). This capability of the normal secretory epithelial cells is the result of their unique ability to accumulate high levels of zinc; which inhibits m-aconitase activity and citrate oxidation [15,16]. Thus, one must recognize that the production and accumulation of citrate is dependent upon and is preceded by the accumulation of zinc in the glandular epithelial cells. Therefore, changes in the level of citrate in the peripheral zone are the result of and indication of changes in zinc levels. This is also evident in table 1 and in figure 1 that show the parallel zinc and citrate levels in prostate. Recent development of in situ magnetic resonance spectroscopy of prostate citrate levels in normal peripheral zone and malignant loci conclusively establishes that citrate levels

are always greatly reduced in malignancy [see reviews 17-19]. The consistency of this citrate relationship now makes magnetic resonance spectroscopy imaging of the prostate gland the most effective and reliable procedure for the identification, localization and volume estimation of malignant loci in the peripheral zone. As revealed in figure 1, and confirmed by numerous other reports, there exists no case in which the malignant loci retain the high citrate levels of the normal peripheral zone glands. These citrate changes revealed by magnetic resonance spectroscopy provide indirect evidence of corresponding changes in the accumulation of zinc, which is the cause of the changes in citrate. This is further verified by the comparative changes in zinc shown in figure 1. The profile of direct measurements of zinc changes associated with malignant prostate tissue strikingly replicates the citrate profile. This is evident despite the fact that these are different studies with different subjects and different stages of cancer. These relationships provide compelling evidence that, in prostate cancer, the malignant cells lose the ability to accumulate high zinc levels; and malignant cells that retain the accumulation of high zinc levels virtually never exist.

The Concept of the Role of Zinc in Prostate Cancer: Is hZIP1 a Tumor Suppressor Gene?

The existence of the zinc and citrate relationships in normal prostate and prostate cancer is irrefutable. How these relationships are involved as factors in the development and progression of prostate malignancy is important to understanding the pathogenesis of prostate cancer. This requires an understanding of the effects of the zinc and citrate-associated metabolism on the requirements of the malignant activities of the neoplastic cells. It is well documented that all tumor cells undergo metabolic transformations that are essential for their malignant existence. It is important to emphasize that these metabolic transformations are not

the cause of malignancy. Malignancy requires the genetic transformation of a sane cell to a neoplastic cell that is endowed with the potential capability of malignancy. The metabolic transformation is essential for the neoplastic cells to manifest their malignant capabilities.

The accumulation of zinc in normal prostate glandular epithelial cells results in two important effects; a metabolic effect and a proliferative effect. Its metabolic effect is the inhibition of citrate oxidation that is essential for the prostate function of production and secretion of high levels of citrate [15,16]; and its inhibition of terminal oxidation [20]. This has a bioenergetic cost in that the inhibition of citrate oxidation results in a ~60% loss of ATP production that would arise from complete glucose oxidation (figure 7). Consequently, zinc-accumulating citrate-producing cells (normal peripheral zone epithelial cells) are energy-inefficient cells. A second effect of zinc is its inhibition of prostate cell proliferation. This effect results from zinc induction of apoptosis in prostate cells (21-24). These are the consequences imposed upon highly specialized zinc-accumulating citrate-producing cells (i.e. normal peripheral zone secretory epithelial cells) in order to achieve their unique function of net citrate production.

Malignant prostate cells do not exist for the specialized function of citrate production and secretion. They must replace the metabolic pathways associated with net citrate production with metabolic relationships that are suitable for their malignant existence. That the malignant prostate cells in situ never exist as zinc-accumulating citrate-producing cells is evidence of the incompatibility of the high zinc accumulation and net citrate production for their existence. Their metabolic transformation to energy-efficient citrate-oxidizing cells that have lost the ability to accumulate zinc provides their metabolic/bioenergetic requirements of malignancy. Also, the apoptotic influence of zinc is eliminated, which permits the proliferation of the malignant cells.

This concept is represented in figure 2. The occurrence of this metabolic transformation is dependent upon the ability of the normal epithelial cells and the inability of the malignant cells to accumulate zinc. The present studies establish that *hZIP1* is down-regulated in the adenocarcinomatous glands. This is consistent with the down-regulation of *hZIP1* gene expression in the African-American male population, which exhibits a higher incidence of prostate cancer. The functional importance of hZIP1 in the accumulation of zinc in prostate cells has been established (10-12). Over-expression of hZIP1 results in increased accumulation of zinc which leads to inhibition of cell proliferation; whereas cells with down-regulation of hZIP1 have decreased cellular zinc levels and increased proliferation. Also the accumulation of zinc in the malignant prostate cells in culture and in vivo (24) results in increased citrate levels.

Consequently, the consistent clinical and experimental evidence strongly implicates the down-regulation of hZIP1 with the lost ability of the malignant cells to accumulate zinc. The existence of hZIP1 insures that prostate cells will accumulate zinc. If ZIP1 is not down regulated in the neoplastic cell, zinc accumulation and its metabolic/energetic and apoptotic effects will prevail; and the neoplastic cell will remain in a pre-malignant dormant state and/or will die. In this concept (figure 2), prostate malignancy requires two essential transformations; the genetic transformation to a neoplastic cell with potential malignant capability; and the metabolic transformation to an energy-efficient citrate-producing cell that has lost the ability to accumulate zinc. Thus, ZIP1 would be a tumor-suppressor gene in prostate cancer. These relationships provide a plausible explanation and expectation for the apparent absence of the identification of malignant prostate glands that exhibit high zinc and high citrate levels.

The focus of this report on ZIP1 is not to imply that other zinc transport processes might not be involved in the altered accumulation of zinc. Rishi et al [13] demonstrated that ZIP1 and

also ZIP2 are expressed in human prostate glandular epithelium. An increase in export of zinc could also decrease zinc accumulation by "true" malignant cells. However no information currently exists concerning the functional role of zinc exporters in prostate cells. Beck et al [25] reported that ZnT-4 was decreased in peripheral zone malignant tissue when compared to normal peripheral zone tissue samples. ZnT-4 is associated with the sequestering of cytosolic zinc into organelles, and is not involved as a plasma membrane zinc exporter. Moreover a decrease in Zn-T4 would not be associated with a decrease in cellular zinc level, even as a secretory process. They also reported that Zn-T1 expression was unchanged in malignant versus normal peripheral zone. Zn-T1 does function as a plasma membrane-associated zinc exporter in some cells and possibly in prostate cells. Hasumi et al [26] reported that Zn-T1 expression was significantly lower in malignant prostate tissue samples when compared to BPH samples, which led them to conclude that Zn-T1 was not likely to be associated with the decreased zinc accumulation in the malignant cells. Consequently, a possible role of altered expression of zinc exporters in the genetic/metabolic transformation of the malignant cells in situ is not evident, but more research is required regarding this issue. We are now investigating the possible involvement of other zinc transporters in prostate malignancy.

Conclusions

The present studies, conducted independently in three institutions, collectively establish the presence of *hZIP1* gene expression, the presence of membrane-associated hZIP1 transporter protein, and the accumulation of cellular zinc in the normal peripheral zone glandular epithelium and in benign hyperplastic glandular epithelium. In contrast, the studies reveal that *hZIP1* gene expression is down-regulated and hZIP1 transporter protein is depleted in adenocarcinomatous glands in prostate cancer. Correspondingly, the cellular level of zinc is also depleted. These

effects occur in early and late stages of malignant development of the peripheral zone. *hZIP1* expression is evident in the malignant prostate cell lines in culture. This leads to the likelihood that the lost expression in the adenocarcinomatous glands is due to an epigenetic silencing of *hZIP1* that occurs under the in situ environment of the peripheral zone. When coupled with the voluminous clinical and experimental evidence, it becomes irrefutable that the development of malignancy in prostate cancer involves an essential metabolic transformation that results in the lost ability of malignant cells to accumulate zinc. Conversely, as long as the capability of high zinc accumulation exists, the neoplastic cells cannot manifest their malignant potential. Consequently, the expression of *hZIP1* that sustains zinc accumulation in prostate cells will prevent the malignant activities and proliferation of the neoplastic cells. This provides a compelling basis for the proposal that *hZIP1* is a tumor suppressor gene in prostate cancer. Consideration of all the clinical and experimental evidence leads to the concept of an important role of zinc and citrate-related metabolism in the pathogenesis and progression of prostate malignancy.

Methods

1. U.Maryland Study

Immunohistochemistry of Human Tissue Sections

Paraffin mounted serial sections of human prostate tissue was used for hZIP1 immunohistochemistry staining. Hematoxylin and eosin staining was used for identification of normal and adenocarcinomatous glands. For immunohistochemistry, slides were dewaxed by incubation in xylene and then rehydrated. Non-specific binding of antibody was blocked by incubation in BlokHen (Aves Labs, Inc.) solution. The slides were washed with PBS, incubated in hZIP1 antibody solution, washed again, and incubated with fluorescein-labeled secondary

antibody solution; and then washed and mounted with anti-fade fluorescent medium (Molecular Probes). For control staining, adjacent serial sections were stained as described above expect that the antibody-depleted and preimmune preparation were used instead of antihZIP1 antibody

Immunocytochemistry of Prostate Cells

PC-3 and LNCaP cells were plated on cover slips. The cover slips were washed with PBS, and the cells fixed in paraformaldehyde solution. The cells were permeabilized by incubation in 0.2% NP-40 solution, washed in PBS, and stained by the procedure described above for immunohistochemistry.

RT-PCR of Human Tissue mRNA. hZIP and GAPDH cDNA were synthesized from total mRNA isolated from human prostate tissue using 1.0 ug of total RNA, reverse transcriptase and random primers (TaqMan7 reagents, Perkin Elmer). hZIP1 and GAPDH fragments were amplified from the cDNA using 1.0 μM forward and reverse primers and 35 cycles. These conditions were shown to be in the quantitative detection range based on the concentration of template DNA. The cloned cDNA for hZIP1 was used as the template DNA in control reactions to determine the specificity of the PCR reactions. The RT-PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining and photographed under UV light. No products were detected without reverse transcriptase. The primers for hZIP1 were 5'-TCAGAGCCTCCAGTGCCTGT-3' and 5'-GCAGCAGGTCCAGGAGACAA-3'

2. The Roswell Park Study

Immunohistochemistry of Human Tissue Sections

Embedded prostatic adenocarcinoma slides that contained both benign prostatic hyperplasia (BPH) and adenocarcinomatous foci were obtained from Roswell Park Cancer Institute. Normal glands and intra epithelial neoplastic foci (PIN) were seen in few cases. Immunohistochemistry

with anti-hZIP1 antibody was performed by standard protocol [27]. Briefly, the slides were deparaffinized. Antigen retrieval was done by heating in 10 mM sodium citrate buffer (pH 6.0) @ 98^0C, incubated in 1% hydrogen peroxide (H_2O_2), blocked with 5% BlokHen with avidin D, incubated with ZIP1 antibody in 5% BlokHen with biotin (Vector Laboratories) at 4^0C over night followed by incubation with Horseradish peroxidase-labeled goat anti chicken IgY secondary antibody in a dilution of 1:200 (AvesLabs, Tigard, Oregon). Color was developed by incubating slides with DAB kit (Vector Laboratories) followed by Hematoxylin counterstaining. Sections were examined with light E600 Nikon microscope. Pictures were taken with Spot advanced soft ware (version. 4.0.1). The appearance of membrane-associated hZIP immuno-positivity of the glandular epithelial cells was used for scoring as previously described [27]. The scores employed were; negative, no positive cells; + <10% positive cells; ++ 10-50% positive cells; +++ > 50% positive cells. The mean scores between groups were analyzed by the Student's t-Test.

3. The ClaflinU. Study

RT-in situ-PCR of Human Tissue Sections

Fresh frozen sections from 38 post-prostatectomy of men with clinical histories of prostate cancer were processed for zinc content analyses and RT-in situ-PCR. RT-in situ-PCR of the frozen sections was performed as described in detail by Rishi et al [13]. 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. Microscopic examination usually reveals cytoplasmic staining for mRNA versus nuclear staining for DNA 22–24. Cell enumeration was performed on coded slides by at least two pathologists.

Determination of Intracellular Zinc Content

The relative intracellular zinc content in situ was determined by utilizing fresh frozen tissues. For this purpose the cells must be biochemically active. The relative concentrations of zinc 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; Newport Green (NPG), and TSQ. The frozen tissues were incubated in 20 ul/section of the zinc indicator cocktail over night and washed in PBS, gently, without disturbing the tissues. The slides were heat-fixed for 10 sec at 104 ^{0}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 high affinity for zinc (Kd~10nM) and a detection limit of ~0.1 nM. The ZN-TSQ positive cells stain red. NPG has moderate zinc-binding affinity (Kd ~1 μM). The ZN-NPG positive cells appear yellowish green. Together, TSQ and NPG provide a relative difference in zinc concentrations in various cell types of the prostate. TSQ has about 2-3-log higher affinity for zinc than NPG, but has detection limit of about 3-log lower than NPG. Therefore, the cells that contain very low concentrations of intracellular zinc appear red and the ones with higher concentrations appear green. The cells with no detectable Zn2+ will appear black or dark blue.

Authors' Contributions

Umaryland Study: RBF and LCC conceived and directed the study, wrote the Umaryland studies, wrote the final manuscript. BM performed ZIP immunohistochemical study. PF provided malignant cells and performed Western blots. **Roswell Park Study:** KS directed the study. MD obtained and conducted analyses of prostate cancer slides. KS and MD wrote the Roswell Park studies. **ClaflinU Study:** AK-D provided human tissue samples, performed histopathology,

made the diagnosis and cataloged the tissues. OB performed the in situ RT-PCR on slides, developed the in situ zinc method, wrote the ClaflinU studies

Acknowledgements

The UMaryland study was supported by NIH grants CA 79903 and CA 71207 (LCC and RBF). The Roswell Park study was supported by grants from the National Institutes of Health RO1-097714 and Elsa Pardee Foundation (KKS). The ClaflinU study was supported by DOD grant DAMD 17-02-1-0233 (OB).

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Figure Legends

Figure 1. Composite of zinc and citrate levels in prostate. The zinc data are taken from Zaichick et al [3] and show the range of zinc levels in resected prostate tissue samples from different subjects. The citrate data are taken from Kurhanewicz et al [33] and show the range of citrate

levels as determined by in situ magnetic resonance spectroscopy imaging of the prostate gland of different subjects. Note the parallelisms in that zinc and citrate levels are consistently significantly low in malignancy; and that no case exists in which the malignant loci retain the high zinc or high citrate levels that characterize normal or hypertrophic glands. The values above each bar are the number of subjects.

Figure 2. The integrated role of ZIP1, zinc, and citrate metabolism in the pathogenesis of prostate malignancy. The normal glandular epithelial cell expresses ZIP1 that permits zinc accumulation, which inhibits citrate oxidation and terminal respiration. Citrate accumulates and coupled ATP production is reduced. A genetic transformation results in a neoplastic cell with potential malignant capability. *ZIP1* expression is silenced by epigenetic factors which eliminates Zip1 transporter and accumulation of zinc in the premalignant cell. The level of cellular zinc decreases which removes the inhibitory effects on citrate oxidation and terminal oxidation. The Krebs cycle is functional and coupled ATP production is increased. The malignant cell is metabolically and bioenergetically capable of manifesting its malignant potential. Additionally, the growth inhibitory effect of zinc is removed, which allows growth and progression of the malignant cell.

Figure 3. **(A)** Immunohistochemical determination of ZIP 1 transporter levels in normal and malignant prostate glands. The strong positive reaction is evident in the normal gland secretory epithelial cells that border the lumen, and is virtually absent in the malignant glands. Note that ZIP1 is not apparent in the stroma. **(B)** RT-PCR of RNA extracted from malignant prostate tissue and benign prostatic hyperplasia. Note the marked decrease in ZIP1 mRNA in the malignant

tissue. **(C)** Immunohistochemical detection of ZIP1 in malignant prostate cell lines. Note the association of ZIP1 with the plasma membrane.

Figure 4. **(A)** Immunohistochemical detection of ZIP1 transporter protein in malignant and nonmalignant loci of a representative prostate cancer tissue section. Note the immuno-positivity of the plasma membrane of BPH and normal glands. The malignant and PIN loci show no detectable ZIP1 so that the plasma membrane of these cells is not visible.

FIGURE 5. Comparative results of ZIP1 immuno-positive glands of tissue sections from subjects described in Table 3. **A.** Summary of glands that exhibited a positive Zip1 reactivity. The number of cases is shown in each bar. **B.** The number of cases in which the glandular epithelium contained cells that exhibited a ZIP1 score >+ (more than 10% of the cells comprising the acini). The differences in A and B between BPH glands and adenocarcinomatous glands are significant, $P<0.01$.

Figure 6. In situ detection of *ZIP 1* expression and zinc levels in normal and malignant glands. **Panel A. *ZIP1* Expression**. In all specimens, the adenocarcinomatous glands exhibit a complete absence of detectable *ZIP1* mRNA in the glandular epithelium (arrows). In contrast the normal glands exhibit a high expression of *ZIP1* (arrows, bright green appearance) in the glandular epithelium; and no *ZIP1* expression in the stroma. The positive control shows the validation of the assay where GAPDH gene segment is amplified by the RT-in situ PCR method. **Panel B. Zinc Levels.** High zinc is represented by Newport Green yellow stain and low zinc is represented by TSQ red stain. The malignant region of the peripheral zone shows a significant

depletion of zinc in the malignant glandular epithelium as exhibited by the red staining (white arrows). The depletion of zinc is evident in early differentiated malignant glands as represented by combinations of red and yellow staining in the glandular epithelial cells. As malignancy advances to the undifferentiated stage, the zinc is further depleted as represented by the dominant red stain and no yellow stain in the glandular epithelium of the adenocarcinomatous glands. The depletion of zinc in the malignant glandular region results in the surrounding stroma showing a higher zinc level (green stain) than the glandular epithelium. In contrast, the normal peripheral zone glands exhibit high zinc levels as represented by the uniform yellow stain and absence of red stain in the glandular epithelium. The stroma surrounding the glands exhibits a lower zinc level as shown by the red stain.

Figure 7. The role of zinc in the pathway of net citrate production in prostate cells. The cellular uptake of zinc by ZIP1 results in accumulation of zinc that inhibits m-aconitase and truncates the Krebs cycle. Citrate accumulates for secretion. The aspartate-glutamate-citrate synthesis pathway is required to provide the oxalacetate for condensation with acetylCoA that is derived from glucose utilization. The bioenergetic consequence of net citrate production is a ~60% decrease in ATP production from glucose utilization.

Table 1. Representative Citrate and Zinc Levels in Prostate

(nmols/gm wet weight)

	CITRATE	ZINC
NORMAL PERIPHERAL ZONE	13000	3000
MALIG. PERIPHERAL ZONE	500-2000	500-900

Table 2. Zinc Levels of Human Prostate Gland		
CITATION	NORMAL	PCA
28*	540	168 (-69%)
29*	517	194 (-62%)
30#	125	46 (-63%)
31#	156	39 (-75%)

Table 3: ZIP1 immuno-positivity of glandular components in tissue sections of confirmed cases of prostate cancer.

Case no.	Grade	ZIP1 IHC score[a]			
		Normal	PIN	BPH	Malignant
1	3	+++		+++	Negative
2	3		+	++	+
3	1		Negative	+	Negative
4	2			+	Negative
5	2			Negative	Negative
6	1	Negative		+++	Negative
7	2	++		+	+
8	1			+++	+
9	1	++		+++	Negative
10	2			+++	Negative
11	1			++	Negative
12	2			Negative	Negative
13	1			++	+
14	1			+	Negative
15	1			+	Negative
16	1		Negative	+	Negative
17	2			+++	+
18	1		+	++	+
19	1			+	Negative
20	1			+	Negative
21	2		+	+++	+
22	1			Negative	Negative
NEG ZIP1 IHC				3/22 (14%)	15/22 (68%)*
SCORE > +		3/4	0/6	14/22	0/22*
MEAN SCORE[b]		1.75	0.6	1.68(1.09)	0.32(0.48)*

[a] Scoring of immunoreactivity was done as follows:
negative, no positive cells; score +, <10% positive cells;
score ++, 10-50% positive cells; score +++, > 50% positive cells

[b] MEAN SCORE: MEAN(SD)
* $P < 0.01$; BPH VS MALIGNANT GROUPS

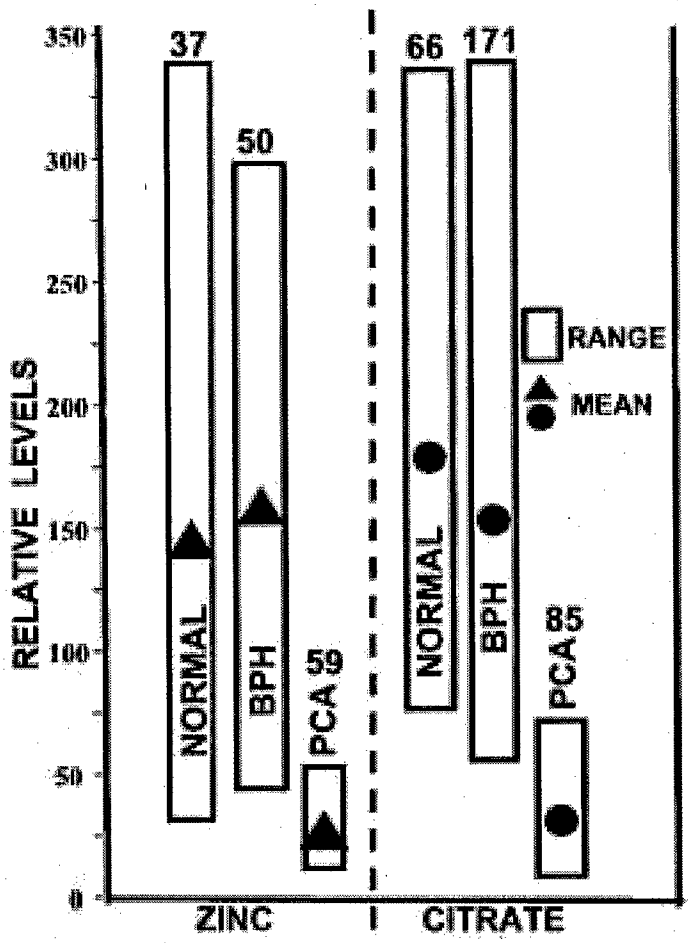

350
300
250
200
150
100
50
0
RELATIVE LEVELS
37
50
59
66
171
85
NORMAL
BPH
PCA
NORMAL
BPH
PCA
RANGE
MEAN
ZINC
CITRATE

Figure 1

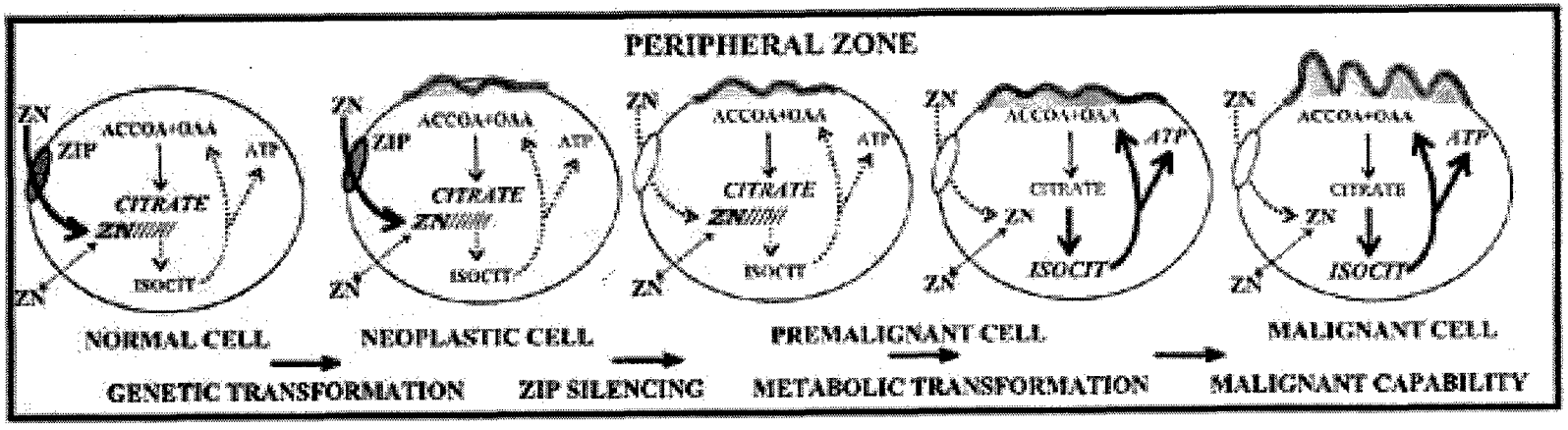
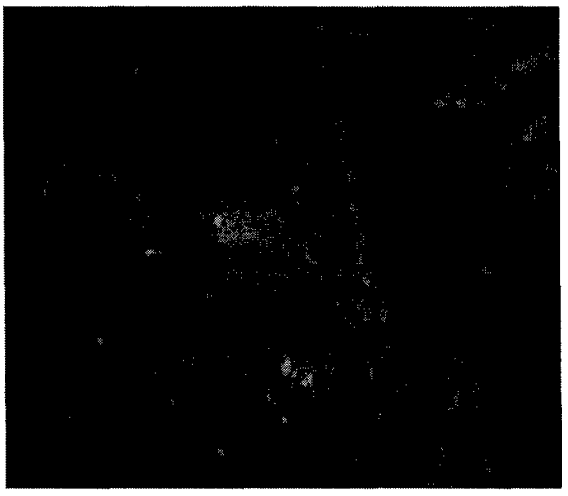


Figure 2

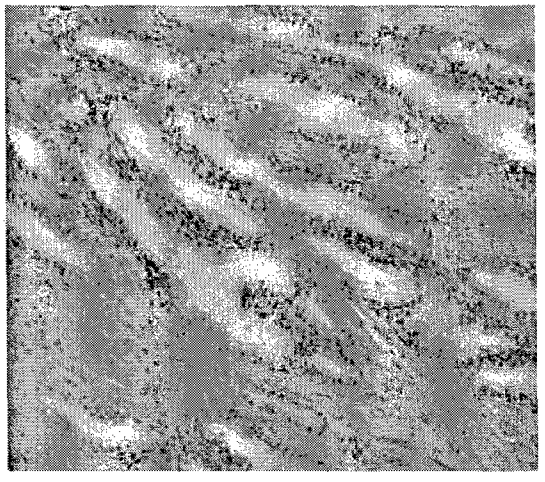
Figure 2

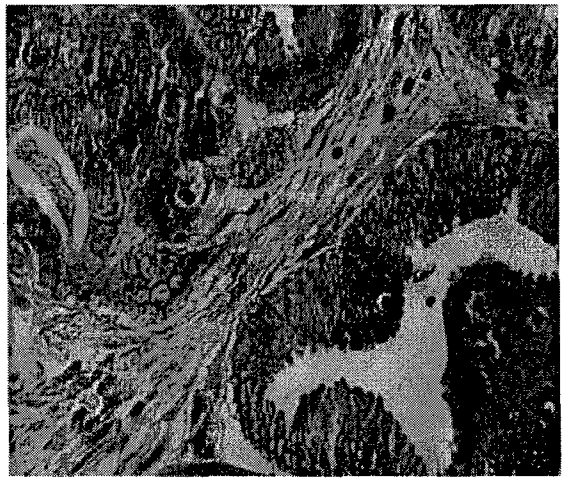
A



Normal

Malignant

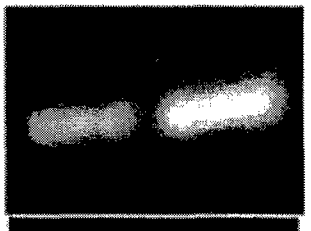




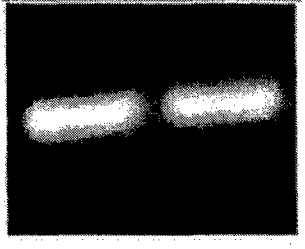
B

PCa BPH

hZIP1

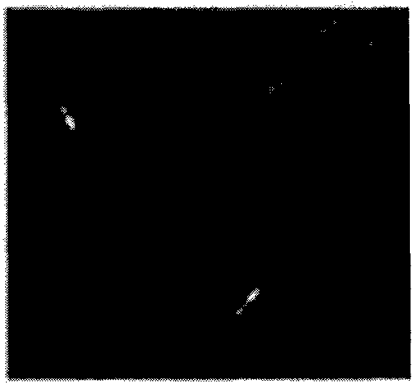


GAPDH



C

PC-3



LNCaP

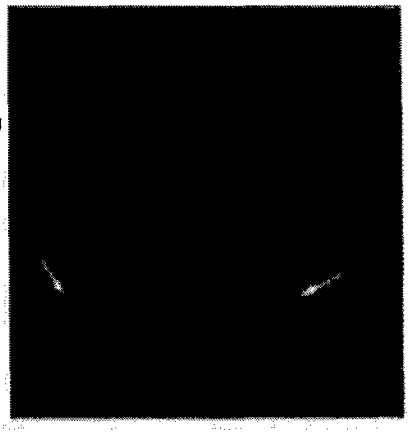


Figure 3

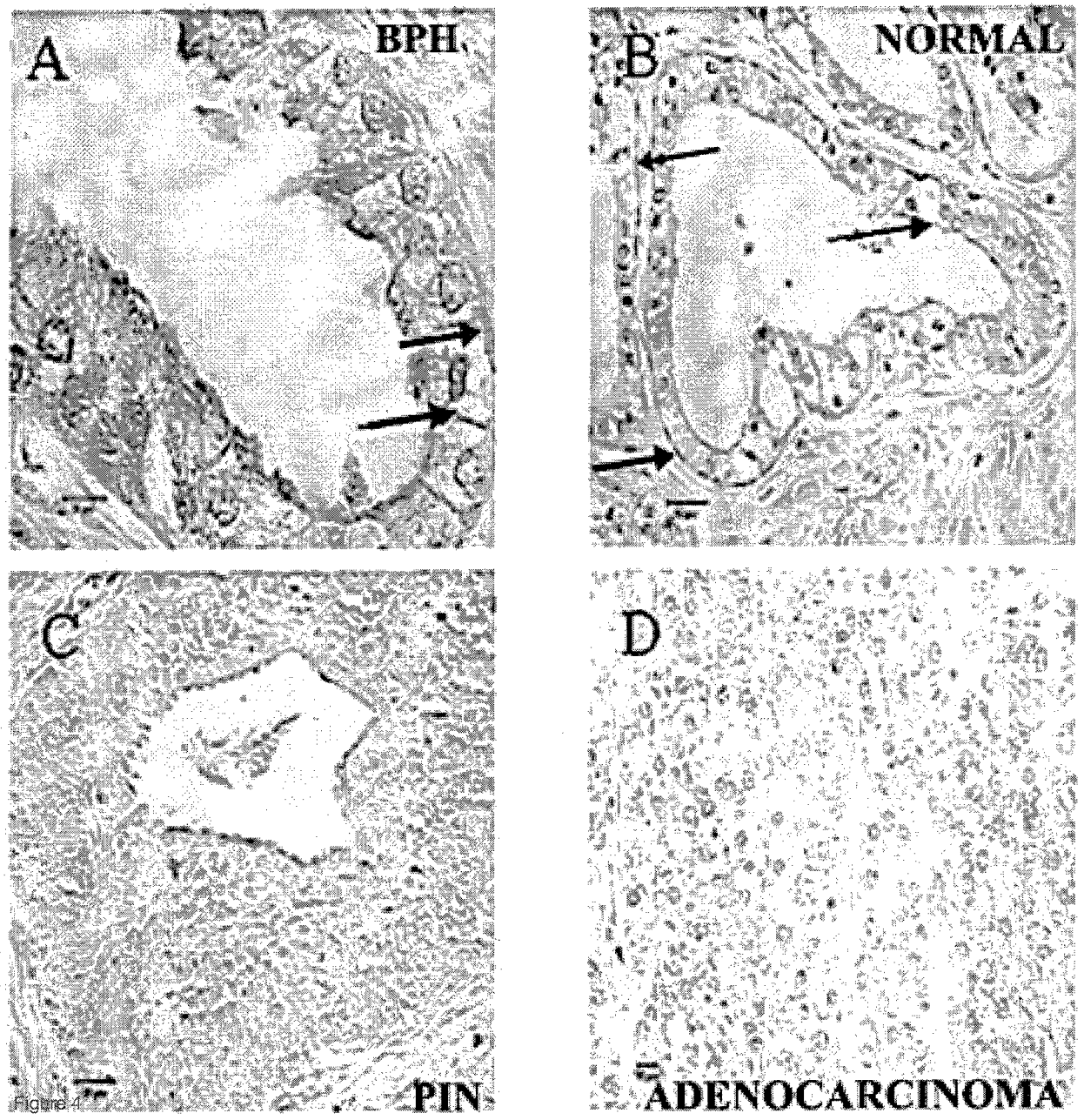


Figure 4

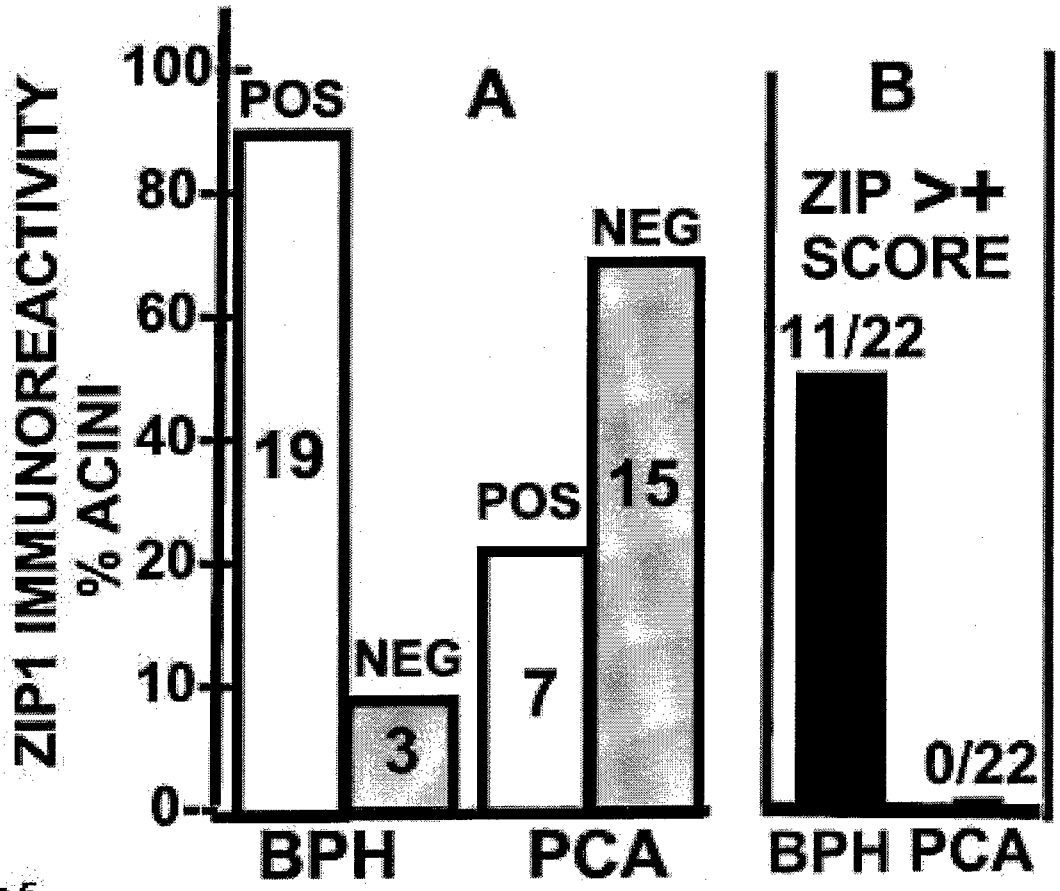

ZIP1 IMMUNOREACTIVITY
% ACINI
100
80
60
40
20
10
0
POS
19
NEG
3
POS
7
NEG
15
A
BPH
PCA
B
ZIP >+
SCORE
11/22
0/22
BPH PCA

Figure 5

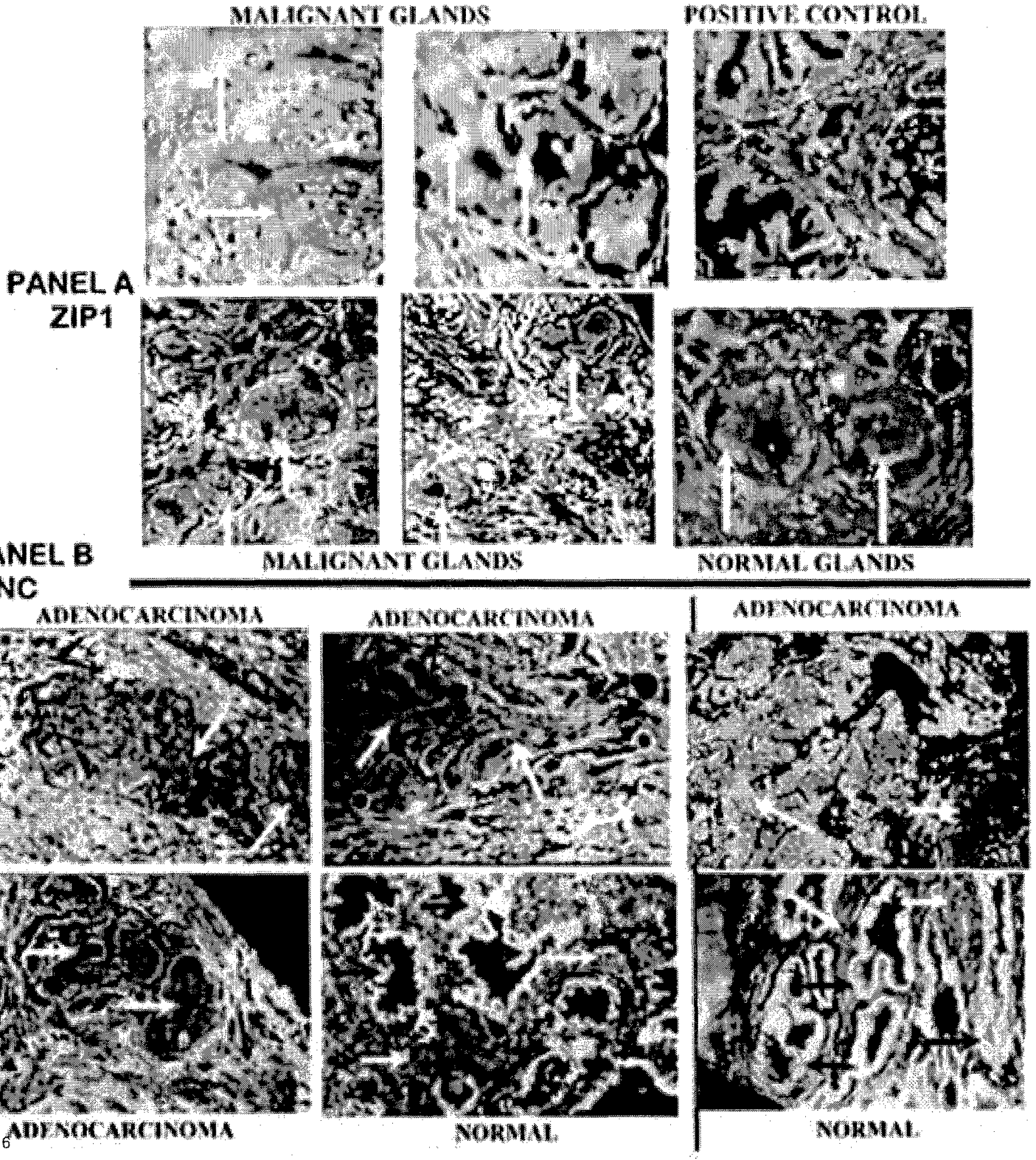
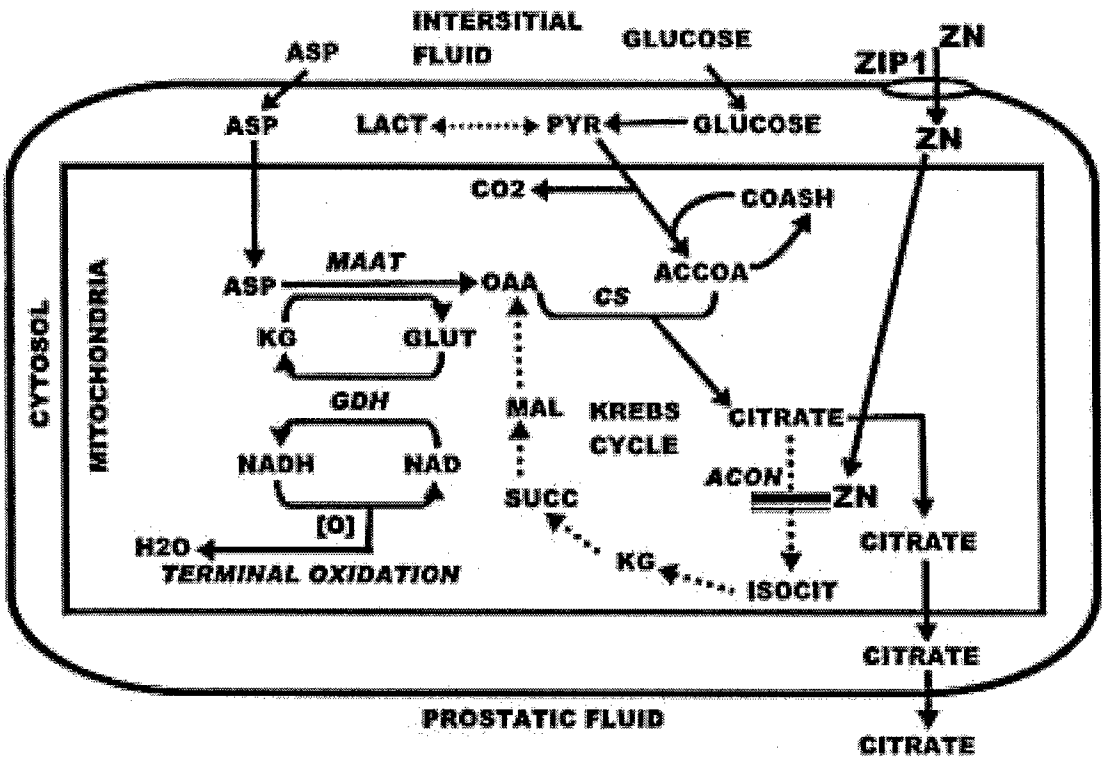

MALIGNANT GLANDS
POSITIVE CONTROL
PANEL A
ZIP1
PANEL B
ZINC
MALIGNANT GLANDS
NORMAL GLANDS
ADENOCARCINOMA
ADENOCARCINOMA
ADENOCARCINOMA
ADENOCARCINOMA
NORMAL
NORMAL

Figure 6



CITRATE OXIDIZING CELLS - MALIG. PROSTATE CELLS

$GLUC + 6O2 \longrightarrow 6\ CO2 + 38\ ATP$

CITRATE PRODUCING CELLS - NORM. PROSTATE CELLS

$GLUC + 2\ ASP + 2\ O2 \longrightarrow 2\ CIT + 2\ CO2 + 14\ ATP$

Figure 7

Medical Hypotheses (2005) 65, 887–892

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Role of zinc and zinc transporters in the molecular pathogenesis of diabetes mellitus

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Received 16 December 2004; accepted 10 February 2005

Summary Diabetes is one of the most common chronic diseases in the United States. An estimated 18.2 million people in the US (6.3%) have diabetes; among them 2.8 million are African Americans (AAs). On average, AAs are twice as likely to have diabetes as European Americans (EAs) of similar age. AAs disproportionately suffer from various diseases in the US. Many of these diseases include hypertension, cardiovascular disease (CVD), diabetes mellitus (DM-β predominantly Type II), and cancers of the prostate and pancreas. A number of risk factors such as smoking, a high fat diet, little physical activity, stress, and meager access to health care have been the subject of numerous investigations. However, the factor of the interaction between genetics and the environment has received very little attention in the scientific community. Of note, the content of zinc in pancreatic β gells is among the highest in the body; however, very little is known about the uptake and storage of zinc inside these cells. We hypothesize that one of the major reason AAs disproportionally suffer from DM (as well as some other illnesses like prostate cancer, CVD and hypertension) is due to their inherent inability to transport appropriate amount of zinc in the crucial cell types that require relatively higher amount of zinc than the other cell types. In this article, we will explore in detail the possible genetic and environmental link between human zinc transporters (hZIPs) and their differential expressions in the islet β cells from AAs as compared to other racial groups, particularly EAs, in both normal healthy individuals and diabetic patients. We hypothesize that the hZIPs play an important role in the development of diabetes, and the main reason AAs disproportionately suffer from DM (as well as other illnesses like prostate and pancreatic cancers, hypertension, and CVD) as compared to EAs may be due the low degree of expressions of the critical zinc transporters in the β cells.

Understanding the molecular events in the pathogenesis of DM with regards to regulation of zinc uptake would be critical to the evaluation of the natural history of diabetes in humans and especially in various racial groups. If a direct link between zinc transport and diabetes can be established, then a special nutritional formula, medication or other intervention might be especially designed to test the ability to decrease the incidence of this disease in DM susceptible groups, particularly in AAs.

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0306-9877/$ - see front matter
doi:10.1016/j.mehy.2005.02.047

Background and significance

Intracellular homeostasis for zinc is achieved through the coordinate regulation of specific transporters engaged in zinc influx, efflux, and intracellular compartmentalization reviewed in [1–3]. The content of zinc in pancreatic β cells is among the highest in the body; however, little is known about the uptake and storage of zinc inside these cells. The high zinc requirement of the β cells relates to each hexameric insulin crystal containing two zinc atoms in insulin granules [4]. When exocytosis of insulin occurs, an insulin granule fuses with the β cell plasma membrane and releases their contents into the circulation [5]. Thus, zinc is secreted along with insulin, thereby requiring the continual replenishment of zinc in the β cells [4,5]. Because free zinc is toxic to a number of proteins in the β cells, zinc must be complexed to the specific zinc binding proteins [6]. There appears to be a complex relationship between zinc and both types of DM, since several complications of DM may be mediated through oxidative stress that is amplified in part by zinc deficiency [7]. Therefore, zinc is an important mediator of insulin storage and secretion, and β cells require highly specialized proteins and signaling system to regulate zinc import, export and subcellular localization and storage of zinc [1–3]. In the human organism, intracellular transport, localization and concentration of zinc is strictly regulated [1–3], and metallothioneins and zinc transporters (*hZIPs*) are the two main components that carry out this function [1,8,9]. The former are involved in storage and trafficking, whereas the later carry out transport of zinc across the membranous structures [1]. The significant clues regarding the uptake of zinc are already accumulating with great speed [1–3,8]. It is now known that in mammals there are at least eight zinc transporters that work in concert to regulate zinc uptake. *hZIP1* to *hZIP4*, are plasma membrane proteins that belong to the superfamily of Zrt/rt-like proteins and are involved in the uptake of zinc from the extracellular environment reviewed in [1,8,9]. All *hZIPs* share the same predicted structure of with six transmembrane-spaning domains and a histidine-rich intracellular loop between helices IV and V [1,2]. *hZIP3* is mainly a brain specific transporter and also is expressed in testes [1]. Kambe et al. [3] have cloned and characterized *hZIP5*. They showed that *hZIP5* was ubiquitously expressed in all tested human tissues and abundantly expressed in the pancreas, prostate, ovary and testis tissues [1,3]. In the human pancreas, *hZIP5* was expressed abundantly in insulin-containing cells that contain zinc at the highest level in the body. They suggested that *hZIP5* plays an important role for transporting zinc into secretory granules in pancreatic β cells. Recently, Chimienti et al. [8] have identified *ZnT-8* as an *hZIP* specific to the pancreas and expressed in β-cells. Because *ZnT-8* facilitates the accumulation of zinc from the cytoplasm into intracellular vesicles, *ZnT-8* may be a major component for providing zinc to insulin maturation and/or storage processes in insulin-secreting pancreatic β cells. *hZIP6* and *hZIP7* have been recently described reviewed in [1,8]. *hZIP6* is unique among other *hZIPs* that it contains serine-rich loop, instead of histidine-rich loop [1–3]. Both *hZIP6* and *hZIP7* are highly expressed in human liver, brain and small intestine [1–3].

β Cells, insulin and types of diabetes

DM represents a heterogeneous group of disorders that have hyperglycemia as a common feature. Generally, DM can be divided into two major groups: one that arises from a defect in insulin production and second, where insulin-producing cells are destroyed [10]. The two major variants of DM: type 1 (T1DM) and type 2 (T2DM) differ in their pattern of inheritance, insulin response and origins. Briefly, T1DM, which accounts for 5–10% of all cases, results from destruction of β cells [10]. The major cause of destruction of the islet cells is autoimmunity (type 1A) and minor cause is idiopathic (type 1B). About 80% of cases are T2DM. The two most important metabolic defects that characterize T2DM are derangement in β cell secretion of insulin and an inability of the peripheral target tissues to respond to insulin (insulin resistance). However, subsequently defective insulin secretion also appears in T2DM. About 10% of the DM cases are due to other reasons (i.e., defective β cells, endocrine dysfunction, infection of pancreas, chemical agents and idiopathic). Most importantly, the long-term complications in blood vessels, kidneys, eyes, and nerves occurs in both types of DM and are the major cause of morbidity and death from DM.

We hypothesize that regardless of etiology of DM, regulation of zinc transporters plays a pivotal role in the pathogenesis of DM. The AA is one of the largest minority groups that suffer disproportionately from DM [10–14]. We have previously shown that zinc transporters are downregulated in AAs as compared to EAs [11]. Our initial hypothesis was based on the data that Africa is a mineral-rich continent and the zinc levels in the water and diet are very high, Africans may have genetically downregulated the degree of their zinc transporters;

otherwise the intracellular zinc levels would have been abnormally high, which reportedly results in serious neurodegenerative and biochemical disorders [11,14–16]. Therefore, individuals of African origin may have a lower capacity to absorb zinc due to their inherent downregulation of zinc transporters when compared to other racial groups [11]. However, when Africans arrived in North America, mostly during the slave trade, they encountered an environment with a relatively lower zinc contents in the water and food, rendering certain cellular types zinc deficient reviewed in [11].

There are two cell types that require extraordinary high levels of intracellular zinc than the rest of the cell types in the human organism – glandular epithelial cells of the peripheral zone of prostate gland and β cells of Langerhans in the pancreas [4,5,11,17–24]. In the case of prostate cancer, a convincing link between low zinc content in the malignant cells of the prostate has been established [11,18–24]. Extensive research has shown that the low intracellular levels of zinc have been associated with the increased incidence of prostate cancer [11,18,19]. It has been well documented that prostate epithelial cells (similar to the pancreas) contain a high concentration of zinc and these levels are significantly decreased in prostate carcinoma relative to normal prostate tissues and are increased in benign prostatic hyperplastic (BHP) tissues [11,18–24]. The prostate epithelium secretes high levels of citrate and proteins that contains zinc reviewed in [22,23]. High levels of zinc and citrate appears to be linked with the normal physiological functions of these cells and a decrease in the intracellular levels of zinc (most likely due to downregulation of various zinc transporters) leads to the initiation of the malignant process reviewed in [22–24].

Here, we hypothesize that certain factors contribute to the down regulation of various zinc transporters in the β cells of the pancreas and decrease its influx initiating a cascade of molecular events leading to DM [11]. Recently, Sprietsma [25] has proposed a mechanism that explains how zinc ions (Zn^{2+}) and nitrogen monoxide (NO), together with glutathione (GSH) and its oxidized form, GSSG, help to regulate immune responses to β cell antigens. NO appears to be able to liberate Zn^{2+} from metallothionein (MT), an intracellular storage molecule for metal ions such as zinc and copper (Cu^{2+}). A Zn^{2+} deficiency can lead to a premature transition from efficient Th1-dependent cellular antiviral immune functions to Th2-dependent humoral immune functions. Deficiencies of zinc++, NO and/or GSH shift the Th1/Th2 balance towards Th2. Via the Th1/Th2 balance, zinc++, NO, MT and GSH collectively determine the progress and outcome of many diseases. He proposed that deregulation of the Th1/Th2 balance is responsible for autoimmune disorders such as T1DM. Extensive research has shown that in T1DM, the Th2 immune system targets four major antigens, insulin, IA-2, glutamic acid decarboxylase (GAD), and heat shock protein 60 (hsp-60). As a consequence, the insulin-producing islets of Langerhans of the pancreas are destroyed. If a child at risk for T1DM by virtue of low zinc or having a first-degree relative with the disease, develops antibodies to insulin, GAD, and IA-2, then the probability of that child developing T1DM in the next five years approaches 100% [1]. Interestingly, according to the recent reports, the plasma zinc concentration in patients with T1DM is significantly lower than in healthy controls and zinc supplement reduces the serum glucose in patients as well as in the animal models of DM [26]. These findings are not universal, and other researchers have found no significant differences in the zinc levels between DM and control subjects [27]. Before we can establish a link between low zinc in β cells and diabetes, it would be pertinent to resolve certain issues. The most important issues that need to be resolved in the area of diabetes are that:

(1) Several immunological factors appear to play a pivotal part in the destruction of β cells in the T1DM [28]. Does the immunological attack on the β cells result from the molecular injury caused by the defective zinc uptake due to downregulation of the zinc transporters, or immune response somehow interferes with the optimal functions of *hZIPs* in the β cells [28]?

(2) Various *hZIPs* are dierentially expressed in human prostate cells [11,19]. Is it similarly true for β cells? The factors that govern the dierential expressions of these transporters may add in the therapy and prevention of DM.

(3) There is evidence that genetic background as well as environmental factors play an important role in the degree of expression of *hZIP1* and *hZIP2* transporters. These two transporters are downregulated in malignant areas of the PZ as compared to surrounding normal cells [19]. Most importantly, expressions of both of these zinc transporters are significantly downregulated in AAs as compared to age and Gleason score-matched white men [11]. Of note, AAs have twice as much incidence of prostate cancer as well as DM than

EAs [4,5,11,17–24,29]. Is dierential expression of various *hZIPs* playing an important role in the molecular pathogenesis of DM?

Role of zinc in DM: zinc supplement can reduce hyperglycemia

The majority of the studies on the potential protective effect of zinc in the development of DM have been performed in animal models of DM [30]. However, few studies carried out in humans are very promising [31–34]. Serum zinc levels status in patients with T1DM is significantly lower than healthy controls [31]. Whether zinc supplementation can prevent the onset of T1DM is unknown. Recent studies have suggested that the generation of reactive oxygen species (ROS) is a cause of β cell death leading to T1DM [33]. In addition, it appears that activation of NF-κB (a ROS-sensitive transcription factor that regulates immune responses) may be the key cellular process that bridges oxidative stress and the death of β cell. Zinc is a known antioxidant in the immune system [32]. Ho et al. [33] have tested whether an increase in dietary zinc can prevent the onset of T1DM by blocking NF-κB activation in the pancreas. The results showed that high zinc intake significantly reduced the severity of T1DM (based on hyperglycemia, insulin level, and islet morphology and numbers) in a well-characterized alloxan and streptozotocin-induced diabetic models. Zinc supplementation also inhibited NF-κB activation and decreased the expression of inducible nitric oxide (NO) synthase, a downstream target gene of NF-κB. It is concluded that zinc supplementation can significantly inhibit the development of T1DM. The ability of zinc to modulate NF-κB activation in the diabetogenic pathway may be the key mechanism for zinc's protective effect. Inhibition of the NF-κB pathway may prove to be an important criterion for choosing nutritional strategies for T1DM prevention. Rouseel et al. [26] have reported the protective effects of supplemental zinc in adult subjects with TIIDM (the predominant type of diabetes in our proposed study), who were given 30 mg/day of zinc gluconate for 6-months. There was a decrease of plasma thiobarbituric acid reactive substances in zinc supplemented group after six months (15%) with no significant changes in the placebo group. Wang and Yang [34] studied 34 TIIDM patients by giving them zinc gluconate for one month and found that supplemental zinc improved the DM condition (lowered their fasting serum glucose).

As compared to zinc supplementation in humans, the animal studies are relatively more clear [35–40]. For example, Simon and Taylor [35] compared the effects of dietary zinc deficiency and zinc supplementation on hyperglycemic control in db/db mice. The dietary zinc supplementation, attenuated hyperglycemia and hyperinsulinemia in db/db mice suggested the roles of zinc in pancreatic function and peripheral tissue glucose uptake. Zinc has been linked to insulin resistance in several studies. Recent studies with diabetic rats have shown that zinc-deficient rats are resistant to exogenous insulin injections [36] and have decreased glucose turnover during a euglycemic hyperinsulinemic glucose clamp [36]. Zinc-deficient rats have impaired glucose tolerance curves compared with pair-fed controls when glucose is administered by the intravenous or intraperitoneal route [36]. Similarly, force-fed zinc-deficient rats (to control for reduced feed intake and altered eating patterns) have impaired glucose tolerance curves despite elevated blood insulin concentrations, normal glucagon concentrations, and normal pancreatic histology [36]. These results suggest that pancreatic insulin secretion is not the limiting factor and indicate an involvement of peripheral insulin resistance. The abnormal glucose tolerance of zinc-deficient rats can be reversed after 1 week of zinc supplement without an increase in caloric intake [36], suggesting that zinc plays an important role in insulin action. However, the preventive role of zinc in DM still needs intensive studies with regards to mechanism of action.

Zinc appears to play a role in modulating insulin receptor tyrosine kinase activity in the skeletal muscle in a genetic mouse model of T2DM. In db/db mice (that has a mutation in leptin receptor gene and is a model of obesity and diabetes) with a metabolic profile (i.e., hyperinsulinemia, hyperglycemia, hyperleptinemia, and obesity) and more closely resembles T2DM than the hypoleptinemia of ob/ob mice (mutation in leptin gene) [40,41]. Others have demonstrated that db/db mice fed a zinc-deficient diet (1 ppm zinc for 4 weeks) have elevated fasting blood glucose concentrations, and that ob/ob mice fed very high levels of zinc (1000 ppm zinc for 4 weeks) or given supplemental zinc in the drinking water have significantly improved hyperglycemia and hyperinsulinemia; however, dietary zinc deficiency and supplementation have not been compared within the same animal model. To test this hypothesis that dietary zinc supplementation would attenuate diabetic signs, Simon and Taylor [35] studied the ob/ob mice by initiating the zinc supplement at the weaning age and continued for a period of 6 weeks to coincide with the period of peak hyperinsulinemia and hyperglycemia in db/db mice [40]. The levels of

zinc for the deficient and supplemented diets, 3 and 300 ppm, respectively, were chosen to induce a marginal zinc deficiency in young mice and to minimize potential adverse effects of high dietary zinc on copper status. Thus, the objectives of the study were: (a) to compare the effects of dietary zinc supplementation and zinc deficiency on hyperglycemia, hyperinsulinemia, and other diabetic parameters in db/db mice and (b) to determine if dietary zinc supplementation or zinc deficiency modulates insulin receptor tyrosine kinase activity in gastrocnemius muscle from db/db mice. These investigators concluded that dietary zinc supplementation attenuated fasting hyperglycemia whereas a marginally zinc-deficient diet exacerbated fasting hyperglycemia in *db/db* mice.

The above discussion clearly shows that zinc has a crucial role to play in the pathogenesis of DM in African Americans. However, more research is needed to throw light on mechanisms of action of different zinc transporters proteins and their transport in and out of cells, especially in tissues like pancreas and prostate gland in humans. A detailed and careful analysis of these transporters may unfold the mystery of diseases like DM and prostate cancer and may increase our understanding of the pathogenesis of these two elusive disorders.

Acknowledgement

Supported in part by a grant from the Department of Army: DAMD 17-02-1-0233.

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Other Support: Omar Bagasra
Current

EPS-044760(Bagasra) 06/01/05 – 05/31/08 10%
NSF EPSCoR
"South Carolina Research Infrastructure Improvement"
The objective of this grant is to build research infrastructure in biotechnology and bioengineering at the University of South Carolina, Clemson University, South Carolina State University, the Medical University of South Carolina and Claflin University.

1P20CA096426-01A1(Bagasra) 07/03/2003 to 06/30/200 25%
NIH/NCI
"Training of Claflin Minorities at USC Cancer Center"
This is a training grant to provide opportunities for training in cancer research to undergraduate students from Claflin University, an HBCU, at the South Carolina Cancer Center laboratories, at USC.

Sub-award 7/01/05 – 6/31/2006 5%
University of South Carolina Institute for Partnerships to Eliminate Health Disparities, W.K. Kellogg Foundation
$141,850
"Differential Expressions of Zinc Transporters in Beta Cells from Various Ethnic Groups
The objective of this study is to detect any differences among various ethnic populations with regard to the expression levels of zinc transporter proteins on Beta islet cells of the pancreas.

HG-14752-05-60 07/01/2005 – 6/31/07 5%
Department of Labor Workforce Initiative Programs
"The Orangeburg-Calhoun Area Biotechnology Consortium Project"
The purpose of this project is to create a job pipeline ladder to produce biotechnology workers at all education/training levels for the state of South Carolina.

Pending

NIH/NCRR (Baynes, John) 06/19/05 to 6/18/10 5%
"SC Infrastructure Networks of Biomedical Research Excellence "
The objective of this grant is to build the biomedical research infrastructure in South Carolina.

Overlap
None

Other Support: Verlie Tisdale
Current
HG-14752-05-60 07/01/2005 – 6/31/07 17%
Department of Labor Workforce Initiative Programs
"The Orangeburg-Calhoun Area Biotechnology Consortium Project"
The purpose of this project is to create a job pipeline ladder to produce biotechnology workers at all education/training levels for the state of South Carolina.

Pending

NIH/NCRR (Baynes, John) 06/19/05 to 6/18/10 10%
"SC Infrastructure Networks of Biomedical Research Excellence "
The objective of this grant is to build the biomedical research infrastructure in South Carolina.

Overlap
None

Other Support: Gemma Geslani
Current

Sub-award 7/01/05 – 6/31/2006 25%
University of South Carolina Institute for Partnerships to Eliminate Health Disparities, W.K. Kellogg Foundation

"African American Public Health Fellowship and Development Program"
The purpose of this program is to recruit and develop African-American students for public health careers, health disparities research, the career development process and appropriate academic preparation needed to prepare for graduate and professional school.

Overlap
This project is complementary to the EXPORT projects proposed but does not overlap.

3235 Differential expression of various zinc transporters in human prostate cancer

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The genetic/molecular basis of prostate cancer is unknown. The most consistent and persistent distinguishing characteristic of a malignant prostate is the inability of the malignant epithelial cells to accumulate zinc. A key factor in the malignant process is the mechanism that results in this inability of the neoplastic prostate cell to accumulate zinc. hZIP1has been identified as an important zinc uptake transporter. Previously, we have shown that gene expression of hZIP1 is downregulated in malignant cells of prostate epithelia. Most interestingly, this downregulation is most prominent in the prostatic epithelia from African American (AA) men as compared to European Americans (EA). Evidence indicates an interplay between the environment and genetics may regulate hZIP1 gene expression that ultimately leads to prostate adenocarcinoma. METHODS: We utilized in situ RT-PCR to assess the relative degree of hZIP1, 2, 3, 4 expressions to localize the zinc transporter expressions at the cellular levels. We measured the relative uptake of zinc by utilizing a unique combination of zinc indicators. RESULTS: Surgical specimens were obtained from patients with assigned Gleason scores ranging from 5 to 8. Relative expression of hZIP-1 and hZnT-4 were significantly downregulated in malignant cells of the specimens, whereas, they were increased in the surrounding normal stromal areas. The degree of downregulation was markedly increased in the specimens from African Americans as compared to European Americans. Similarly, the relative zinc uptake was prominently decreased in the malignant epithelial cells as compared to the stroma. In addition, the relatively lesser degree of downregulations was observed for hZIP-2 and hZnT-3. CONCLUSIONS: Evidence indicates an interplay between the environment and genetics may regulate various zinc transporters and a significant decease in the gene expressions of hZIPs that ultimately leads to prostate adenocarcinoma. Furthermore, our data provides an explanation of why AA men have twice as much incidence of prostate cancer than EA men.

Citation format: Proc Amer Assoc Cancer Res 2005;46:3235.

3204 Effects of various steroid hormones on intracellular zinc uptake in prostate cancer cell lines

Dyantha Moody, Omar **Bagasra** , Meaghen Ashby, Naomi Kariuki, Jenica Hemingway, Frank Gooden, Andre' Kajdacsy-Balla. *Claflin University, Orangeburg, SC and University of Illinois, Chicago, IL.*

The genetic/molecular basis of prostate cancer is unknown. The most consistent and persistent distinguishing characteristic of malignant prostate is the inability of the malignant epithelial cells to accumulate zinc. A key factor in the malignant process is the mechanism that results in this inability of the neoplastic prostate cell to accumulate zinc. hZIP1 has been identified as an important zinc uptake transporter. We have shown that gene expression of hZIP1 is downregulated in malignant cells of prostate epithelia. This project we compared the effects of varying concentrations of steroid hormones on the uptake of zinc by different prostate tissue cell lines. The intracellular zinc concentration in situ, was measured using the zinc indicator dye, Newport Green DCF, (Molecular Probes, Eugene, OR). In our studies, three steroid hormones-testosterone, β-estrodial and prolactin- at varying concentrations, ranging in concentration from .125 nM to 1.22 x 10^{-4} nM, and were tested on three different cell lines, each exhibiting different characteristics with regards to their dependency on various hormones. The three cell lines were: 1.) LnCAP, (ATCC # CRL-1740), an androgen-dependent epithelial prostate carcinoma isolated from the supraclavicular lymph node, 2.) PC3, (ATCC # CRL-1435), an androgen dependent epithelial prostate andenocarcinoma isolated from bone marrow tissue, and 3.) RWPE, (ATCC # CRL-2221), prostate epithelial cell line from normal tissue and transformed with HPV-18. After exposure to the three different hormones overnight, the zinc uptake of the prostate cells was assayed by utilizing zinc indicators. The intracellular levels of zinc were measured by an ELISA based reader and by microscopic visualization. Our analyses show that there is a complex interplay between various hormones with regards to zinc uptake. Implication of a potential therapy or prevention for prostate cancer utilizing a zinc supplement plus a combination of hormones will be presented.

Citation format: Proc Amer Assoc Cancer Res 2005;46:3204.