ACTA SCIENTIFIC CANCER BIOLOGY

Volume 2 Issue 9 November 2018

Review Article

Energy Dispersive X-Ray Fluorescence Zn/Fe Ratiometric Determination of Zinc Levels in Expressed Prostatic Fluid: A Direct, Non-Invasive and Highly Accurate Screening for Prostate Cancer

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Received: September 21, 2018; **Published**: October 10, 2018

Abstract

Prostate specific antigen (PSA) does not provide the reliability that is required for the accurate urology screening of prostate cancer (PCa). Consequently, there has been a major focus and search for a simple, rapid, direct, preferably non-invasive, and highly accurate biomarker and procedure for the urology screening for prostate cancer. Virtually all PCa cases exhibit a marked decrease in zinc in prostate tissue and in prostatic fluid. This is a hallmark "signature" clinical characteristic for all prostate cancers, which provides the clinical basis for zinc screening of PCa. Energy dispersive x-ray fluorescence (EDXRF) of zinc levels in expressed prostatic fluid (EPF) provides > 90% accuracy for the identification of prostate cancer vs normal/benign prostate. An energy dispersive x-ray fluorescence (EDXRF) Zn/Fe ratiometric analysis of expressed prostatic fluid (EPF) can provide > 90% accuracy for the identification of prostate cancer vs normal/benign prostate. This will be achieved by direct EDXRF analysis of a "drop" of EPF directly deposited on a filter paper disc during the urology digital rectal examination of the subject. Interfering and confounding conditions that besiege PSA do not exist in the EDXRF Zn/Fe radiometric analyses. This report reviews the basis for zinc analysis for PCA, provides the supporting evidence that EDXRF Zn/Fe ratiometric analysis of EPF will provide a simple, rapid, direct, non-invasive, and highly accurate biomarker and procedure for the urology screening for prostate cancer.

Keywords: Urology Screening; Prostate Cancer; Zn/Fe Ratiometric Analysis; Energy Dispersive X-Ray Fluorescence; Expressed Prostatic Fluid

Introduction

Stamey *et al*. state in their 2004 report [1] "The prostate specific antigen era in the United States is over for prostate cancer… the relationship of serum PSA in the last 5 years rests exclusively with benign enlargement of the prostate... We urgently need a serum marker that reflects prostate cancer in the current PSA range of 2 to 10 ng/ml... In the meantime, what are we to do in the face of such massive, unwarranted PSA screening".

Since then the issue has continued to be debated with recommendations that PSA be eliminated for "routine" urologic screening; or employed for specific cases; or continued to be employed in the "routine" urology screening procedure [2-4]. Nevertheless, routine PSA testing, in conjunction with digital rectal examination (DRE) remains the major urological screening of prostate cancer; largely due to the absence of a more highly reliable biomarker and procedure as an acceptable alternative to PSA.

This also impacts the decisions regarding the performance of prostate biopsies; of which there are more than one million procedures/year in the U.S. Of these, ~80% are reported as negative for malignancy; and with many being "false" diagnoses; and biopsy infections pose another issue [5].

Consequently, there has been a major focus and search for a simple, rapid, direct, preferably non-invasive, and highly accurate biomarker and procedure for the urology screening for prostate cancer. The major direction of most investigations has been identification of blood plasma biomarkers and various prostate tissue

biomarkers; none of which has achieved the aforementioned requirements.

It has been clinically established that markedly decreased zinc concentration in prostate cancer tissue and prostatic fluid, compared to normal and benign prostate, exists in virtually all cases of PCa; and, thereby constitutes the most consistent hallmark signature identification of PCa. No corroborated reports exist that demonstrate PCa prostate malignancy with the high zinc concentration that exists in normal prostate. The decrease in zinc is an early event in the development of malignancy and persists in progressing malignancy. These relationships offer the opportunity for an accurate specific biomarker for screening of prostate cancer, which will not be compromised by the confounding conditions that besiege PSA or other factors.

Energy dispersive x-ray fluorescence (EDXRF) determination of zinc levels in expressed prostatic fluid (EPF) and in prostate tissue sections provides a direct, specific, and > 90% accuracy in the identification of PCa versus normal/benign prostate. EDXRF directly determines the zinc levels in EPF samples that can be obtained during the DRE that is routinely performed during a urology examination.

In this report, we review the relevant background and evidence of the zinc relationships in normal prostate and prostate cancer; along with the successful identification of PCa with EDXRF analyses of zinc concentration in expressed prostatic fluid and prostate biopsies. We introduce and describe the EDXRF Zn/Fe ratiometric analysis of EPF for the direct, rapid, and highly accurate screening for PCa, which can be performed during the "routine" urology DRE procedure; and with immediate determination of presence or absence of PCa. Thus, the potential now exists that meets all of the requirements for the highly accurate screening for PCa; and which will far exceed the reliability of PSA.

For expanded background, additional descriptions and references of the above relationships, we refer the reader to our earlier reviews [6-10].

The concentration and role of zinc in normal prostate

The human prostate gland is composed of the peripheral zone (~75%), the central zone (~20%), and the periurethral region (~5%). The peripheral zone is mainly responsible for the major function of the prostate gland; i.e. the production and secretion of prostatic fluid that contains an enormous concentration of citrate (the purpose of which remains speculative). This is achieved by the specialized zinc-accumulating acini epithelial cells, in which their m-aconitase activity is inhibited by zinc; so that Krebs cycle citrate production is increased for secretion into prostatic fluid along with zinc secretion. As such, the composition of the prostate gland and prostatic fluid includes high levels of zinc and citrate, and changes in zinc and citrate concentrations generally occur in parallel. Table 1 presents the typical concentrations of zinc in prostate.

Peripheral zone- Normal	2000-4000
Peripheral zone- PCa	200-900
Central zone - Normal	800-1000
Central zone - BPH	700-4000
Other soft tissues	100-500
Prostatic fluid	7000-9000
Prostatic fluid PCa	800-1000
Blood plasma	13-17

Table 1: Typical zinc levels in prostate (nmols/gm).

Decreased zinc occurs in all cases of prostate cancer

More than thirty clinical reports over the past ~sixty years have conclusively established that zinc is markedly decreased in PCa compared to normal and BPH tissues. In nineteen population studies of zinc levels in prostate tissues, all reported that zinc is markedly decreased in PCa tissue compared to normal and BPH tissues. In table 2 we normalized the zinc data in the nineteen reported studies (based largely on [11], which shows that the zinc concentrations are markedly decreased (68% and 76%) in PCa tissues compared to normal and BPH tissues, respectively; with a highly significant statistical difference. Since the zinc levels in the normal and BPH tissues were not statistically different; they comprise the non-cancer group to compare with the PCa group and provides $P < 0.001$ for the decreased zinc in PCa.

Normal		BPH	Norm + BPH	PCA
Mean	598.71*	801.29*	765.72*	194.21
SEM	100.47	101.47	72.36	35.90
% Change	-68	-76	-75	

Table 2: Relative zinc levels in prostate tissues compiled from 19 reports.

*P < 0.0001 versus PCA,

% Change = PCA Zn decrease vs Normal and BPH.

This is a striking consistency when one considers all of the variables and confounding conditions within the populations represented in those studies; such as: differences in the stages of PCa; phenotypic and genotypic difference of the PCa; differences in the ages of the subjects; differences in accompanying conditions of the subjects ; differences in the collection and treatment of the prostate tissues; differences in the zinc assay procedures and calculation of tissue zinc concentration; and other variables.

In addition, several reports of in situ zinc staining have consistently demonstrated that the zinc decrease in the PCa tissues is associated with the loss of zinc in the malignant cells as compared to the high zinc concentration in the normal and benign acinar epithelium (figure 1) [7-9]. The inability of the malignant consistently demonstrated that the zinc decrease in the PCa cells to accumulate the high zinc levels results in the marked tissues is associated with the loss of zinc in the malignant cells as decrease in the zinc concentration of the secreted prostatic fluid.

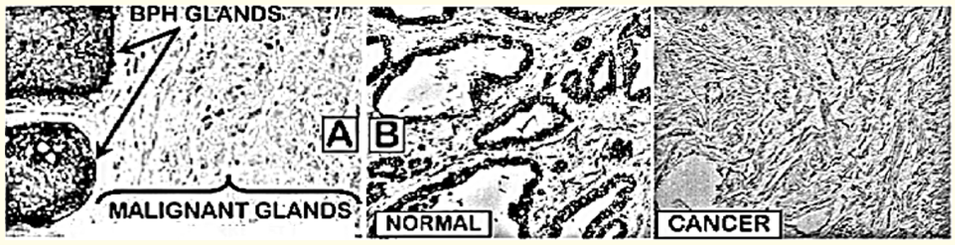

Figure 1: Dithizone zinc stain of human prostate tissue sections.
A Gyorkey., *et al*. [15] BPH acini epithelium with dense zinc stain and surrounding malignancy with low zinc staining. B. Peripheral zone tissue section showing normal acini epithelium with dense zinc stain and malignancy with minimal zinc stain [7].

Thus, the extensive clinical evidence has established that zinc is always markedly decreased in PCa compared to normal/benign prostate; and there are no confirmed or corroborated case in which the concentration of zinc (and citrate) in malignancy is higher than the zinc concentration in the corresponding normal tissue. Decreased zinc, along with citrate, presents the hallmark "signature" clinical characteristics for all prostate cancers; which is an important clinical consideration for any factor that is to be employed as the basis for urology screening of PCa.

The decreased zinc is an early event in the development of malignancy: How and Why?

The decrease in zinc is an early event in transformation of the normal cells to malignancy [6-9]. It occurs during the oncogenic transformation of the normal cells to malignancy. It is evident during the development and progression of premalignancy (PIN; prostate intraepithelial neoplasia) leading to the early grade highly-differentiated malignant cells and persists in progressing malignancy. The decrease in zinc levels results from the downregulation of the functional zinc uptake transporter, ZIP1. This is a required early transformation to prevent the uptake and accumulation of the zinc levels that exist in the normal cells but are cytotoxic in the malignant cells. Thus, the decrease in zinc identifies the early development of malignancy as well as the progression of malignancy, which is why all cases of prostate malignancy exhibit decreased zinc.

EDXRF identification and confirmation of decreased zinc in PCa

Schrodt *et al*. [11], employing x-ray fluorescence, identified a 90% decrease in zinc in cancer tissue sections compared to non-cancer sections, which was confirmed by in situ zinc staining. Zaichick *et al*. [12] employed x-ray fluorescence in an extensive study of prostate biopsy samples that demonstrated a zinc decrease of > 85% in PCa versus noncancer prostate; which provided an accuracy, sensitivity and specificity of 98% for identification of PCa. Their results represented in figure 2 show that none of the 59 PCa cases exhibited a zinc concentration that approached the mean concentration for normal/benign prostate. Cortesi., *et al*. [13] utilizing EDXRF also reported the consistent decreased zinc in PCa biopsies.

Figure 2: X-ray fluorescence determination of zinc concentrations of prostate biopsy samples (modified from [12]).

Zaichick., *et al*. [14] with EDXRF analyses of EPF provided compelling evidence that the concentration of zinc is significantly decreased in EPF samples from PCa subjects compared to noncancer subjects (Figure 3). Notably, as in figure 2, none of the PCa cases exhibited an EPA zinc concentration that approached the mean concentration of the non-cancer groups. The statistical analysis of the noncancer groups vs the PCa group provided a $P < 0.0001$; Based on these results, an extended population study will likely corroborate the accuracy of EDXRF zinc analyses for identification of PCa.

Figure 3: EDXRF determination of zinc concentrations in expressed prostatic fluid. Modified from [14].

Thus, EDXRF corroborates the extensive clinical evidence that has established that zinc is always markedly decreased in PCa compared to normal/benign prostate; and there is no confirmed or corroborated case in which the concentration of zinc in malignancy is the same as or higher than the zinc concentration in the corresponding normal tissue.

It will be necessary to extend and validate the reported EDXRF zinc studies with a large population study that includes normal, benign, and PCA subjects in order to establish the representative full ranges of zinc concentrations for cancer vs noncancer subjects. Those data will provide the scatter-gram that is required to apply the statistical analyses for the accuracy and reliability for the screening of PCa.

EDXRF Zn/Fe ratiometric assay of EPF for accurate screening of PCa: Supporting evidence

EDXRF analysis offers important advantages for zinc measurements of expressed prostatic fluid. It provides direct determination for zinc; and thereby, preserves the sample for pursuant and additional analyses. No reagents or treatments of the EPF samples are required. EDXRF provides a spectrum of elements in EPF, which can be simultaneously determined and referenced to zinc. The EPA sample can be directly collected on a filter paper disc during the DRE, and the EDXRF analysis conducted during the urology examination.

However, EDXRF measurements of zinc have determined the concentration of zinc in the samples, which requires the necessity of collecting and processing the sample to establish the units of concentration; such as volume, dry weight, etc. This imposes the necessity of collecting sufficient EPF sample, withdrawing an accurate aliquot volume, and transferring it to an appropriate platform for EDXRF analysis; thereby introducing potential errors and variables. Such conditions are incompatible as a rapid, direct, accurate procedure for screening of PCa during a urology examination.

In order to eliminate these variables, and to employ the advantages of EDXRF, and to perform a direct assay independent of EPF volume, we initiated studies of the plausibility of developing an EDXRF ratiometric approach. This led to the EDXRF Zn/Fe ratiometric analysis of EPF based on the following studies. We employed EPA samples from 39 subjects (15 PCa; 24 benign), which had been directly deposited on special filter paper discs. We subjected the samples to direct EDXRF spectral analyses.

The spectra of these samples demonstrated that the EDXRF counts for zinc decreased in PCa samples versus benign samples; whereas Fe counts were unchanged relative to zinc counts for PCa and noncancer samples. Therefore, changes in Fe counts will reflect other differences among EPF samples on the filter paper discs; and will serve as a reference for differences in the EPF zinc levels.

Figure 4 shows spectra for three samples that included one PCa sample and two benign samples. Based on the Zn spectrum CPS for the area under the peak, the Zn concentration in the PCa sample is lower than the benign samples. The Fe spectra are similar for the three samples; the volumes and other conditions of the EPF samples were very similar in respect to any impact on the zinc status in PCa vs noncancer samples. As, such the Zn/Fe ratios and the zinc changes will give the same results.

Figure 5 shows EDXRF spectra of a PCa and a benign EPF sample. Based on the counts for the Zn spectrum. PCa exhibited 55% lower zinc compared to the noncancer sample. However, the Fe counts in the two samples were also changed; so that the Zn/Fe ratio of 0.08 results in a 92% decrease in zinc in the PCa sample compared to normal.

Based on this relationship, we proceeded to determine EDXRF spectra on the blinded 39 EPF samples, from which the Zn/Fe

Figure 4: EDXRF spectral analysis of three expressed prostate fluid samples that exhibit different zinc levels and unchanged Fe counts.

Figure 5: EDXRF spectral analysis of zinc levels in a benign and a PCa expressed prostatic fluid sample that exhibit different Fe counts.

ratio was calculated for each sample (Figure 6). The Zn/Fe ratios resulted in 80% accuracy for identification of PCa. The statistical analysis for PCA vs benign samples was highly significant with P=0.0065. Thus, the Zn/Fe ratiometric direct analysis is more accurate and reliable when comparing different samples and eliminating the necessity of determining the zinc concentrations.

We must add some qualifying information regarding this study. The instrument that was available to us for these studies was a 2005 portable EDXRF unit designed for industrial application; and presented conditions that were not consistent with more optimal

Figure 6: Results of EDXRF Zn/Fe ratiometric analyses of expressed prostatic fluid samples from 15 PCa and 24 benign subjects.

requirements for EPF analyses. For example, the collimator window is fixed at 1 cm x 2 cm, which is too large for the volume of EPF collected on the filter paper discs. This imposed high background counts that have to be corrected; and it limited the analysis of multiple fields within the EPF sample. The sensitivity of the instrument was relatively low, which then required lengthy count time. The software was also limited relative to data collection and calculations for the ratiometric analyses. Despite these limitations, an 80% accuracy was achieved for the EPF Zn/Fe ratiometric identification of PCa, even with this small population of EPF samples These and other limitations no longer exist in current EDXRF instruments, which will accommodate the requirements for direct, sensitive, and highly accurate ratiometric analyses of EPF samples.

The EDXRF Zn/Fe ratiometric analysis of EPF samples for routine screening of PCa during urology examination

The routine urology screening for PCa has generally included the invasive PSA testing; which is conducted in the urology facility during the examination or requires the subject to have the PSA test performed by a blood chemistry facility. The EDXRF Zn/Fe metric analysis only requires a drop of EPF directly deposited on a filter paper disc, which is obtained during the digital rectal examination. The results are then immediately available from the analysis that is conducted with an appropriate EDXRF instrument in the urology facility. This is a noninvasive and safe procedure; requires no

additional reagents or sample preparation; the EPF sample is preserved and available for storage and Zn measurements simultaneously with subsequent EPF samples from the patient; or the EPF sample can be used for assays of other components. All of these advantages including the elimination of BPH as a confounding condition, and along with a > 80% accuracy for identification of PCa, favor the EDXRF Zn/Fe ratiometric approach for the replacement of PSA.

The clinical factors that impact the reliability and accuracy of the EDXRF Zn/Fe ratiometric analysis of EPF samples

The major advantage of employing EPF zinc levels is that all cases of PCa exhibit significantly decreased zinc levels. However, it must be recognized that the decrease in zinc also exists in premalignancy. Therefore, the correlation of a decreased EPF zinc level with a prostate gland biopsy could be concluded as a "false positive" result; but that would be a diagnostic error. Unfortunately, it has not been widely recognized among the urology, oncology, pathology community that decreased zinc exists in the early development of malignancy, which precedes the histopathological appearance of malignancy; and it persists during progression of malignancy and in advanced prostate cancer. In their report of EDXRF analysis of zinc levels in 8,323 noncancer segments and 669 cancer segments in biopsy cores from 440 noncancer and 158 cancer cases; Cortesi *et al*. [13] concluded: "Zinc depletion in the prostate peripheral zone is the basis for a novel, noninvasive PCa detection… the zinc depletion occurs not only in the cancerous tissue segments but also, though less pronouncedly, in the non-cancer components surrounding the lesion. This observation is consistent with the conclusions of Costello., et al. that the zinc depletion is an early step in the cancer proliferation process and that zinc depletion precedes the transformation of cells from normal to cancerous type. It is well possible that although PCa has not been observed by the pathologist in these regions, the cellular precursor for its appearance is already present…"

Presently, many and likely most urologists do not and have not collected EPF during the DRE examination. This imposes the issue of the feasibility of most urologists for collecting a drop of EPF. Since many urologists have successfully obtained EPF, it seems likely that most urologists can achieve this capability from most patients. Therefore, this should not pose a deterrent for the incorporation of the EPF EDXRF Zn/Fe ratiometric for identification of prostate cancer.

Conclusions

There is a critical need for a highly reliable, accurate, simplified biomarker and procedure for the screening for prostate cancer; especially during the urology examination of patients, as a replacement or adjunct for PSA.

The relationships of decreased zinc in malignancy in all PCa cases provides the basis for a highly reliable, accurate, simplified biomarker and procedure for the screening of prostate cancer in prostatic fluid.

EDXRF provides an accurate, specific, and sensitive zinc measurements for EPF and biopsy samples. The EDXRF Zn/Fe ratiometric approach provides a direct identification of PCa of EPF and biopsy samples without any alteration or processing of the sample; which can be preserved for additional analyses. This, along with the higher accuracy than PSA (~90% vs ~65%), dictates that EDXRF Zn/Fe ratiometric procedure should be developed and employed for the screening of PCa. Unlike PSA, BPH is not a confounding factor for EDXRF determination of PCa.

The medical community (especially urologists, pathologists, and oncologists) should recognize the important established prostate clinical zinc relationships; and support its development for the screening of PCa. This is in the best interest of the medical community, and in the health and welfare of the public.

Acknowledgement

Studies of L. Costello and R. Franklin cited in this paper were supported by NIH grants CA79903, CA093443, CA71207.

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