

Analysis Expression of ZIP1 and Caspase-3 Protein in Adenocarcinoma of the Prostate

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Abstract

Objective: Carcinogenesis of adenocarcinoma of the prostate occurs due to dysregulation of zinc level within the cells. Intracellular zinc molecules influx is regulated by a transporter protein ZIP1, whose non-presence is predicted to inhibit apoptosis, thus leads to the development of prostate adenocarcinoma. **Methods:** This study was aimed to analyze the correlation of ZIP1 and Caspase-3 expression in prostate adenocarcinoma on its grading as represented by Gleason Score. This was a cross-sectional, retrospective analytical study on 31 formalin-fixed, paraffin-embedded tissue that meets inclusion criteria. The specimen was stained using the immune-histochemistry technique for ZIP1 and Caspase-3. Protein expression of each case was counted using ImageJ analysis. Gleason score was acquired as secondary data from the cases's reports. The correlation of their expression with respect to Gleason score was analyzed with Pearson's correlation using SPSS 11.5. **Results:** Mean expression level of ZIP1 and Caspase-3 in prostate adenocarcinoma were 35% and 33%, respectively. There was a significantly positive correlation between ZIP1 and Caspase-3 expression ($r = 0.379$; $p = 0.018$). However, their correlation was stronger in intermediate-grade group ($r = 0.73$; $p = 0.01$) and the correlation was much weaker in high-grade group ($r = 0.04$; $p = 0.48$). **Conclusions:** There was a positive correlation between ZIP1 and Caspase-3 expression in prostate adenocarcinoma.

Keywords: ZIP1, Caspase-3, Gleason, apoptosis, adenocarcinoma, prostate

Introduction

Prostate cancer is one of the most common malignancy occurring in mention the worldwide, with increasing incidence especially in developing countries and is often diagnosed at an advanced stage.¹ The incidence of prostate cancer in Indonesia ranks the third after lung and colon cancer.² Because of highly incidence of prostate cancer in Indonesia, searching the best therapeutic method for early detection, diagnosis, and treatment of prostate cancer needs to be done.

The development of prostate cancer occurs due to changes in the characteristic of cell proliferation and apoptosis. Normal prostate gland produces fluid, prostatic secretions that contain a variety of essential cell proliferation and apoptosis elements such as zinc, citric, and prostate-specific antigen (PSA) biomarkers.³ The Accumulation of intracellular zinc influenced zinc transporters in the plasma membrane and intracellular membranes. ZIP1 proteins that contained in the basolateral membrane of the epithelial prostate is a major transporter of zinc into the cell carrier. Some research report ZIP1 transport activity and high accumulation of zinc have influence tumor suppressor activity guard cells undergone plastic malignancies.⁴

Based on the various references, it was mentioned that there is a relationship between protein expression ZIP1 as the gateway of zinc ions into cells that spur apoptosis and Caspase-3 activation as executor of apoptosis protein in prostate adenocarcinoma histological grade Gleason score system. This study aims to determine the role of transporter proteins in the process of development of malignancy ZIP1 prostate cancer. Also, this study also seeks to analyze the level of protein expression and activation ZIP1 caspase-3 as well as the correlation both in prostate adenocarcinoma tissues with different Gleason score.

Methods

The study design was a cross-sectional retrospective analytical study design. The research subject is a paraffin block prostate adenocarcinoma which had been diagnosed by a medical anatomical pathology doctor specialist with complete data Gleason score (GS). All data contain in the archive department of pathology-anatomy, Faculty of Medicine, Universitas Indonesia and Cipto Mangunkusumo Hospital (RSCM). The specimen was stained using the immune-histochemistry technique for ZIP1 and Caspase-3. Based on the

Gleason score, subjects were divided into five groups: GS6, 7GS, GS8, 9GS, and GS10.

Calculation of sample size. The sample size was calculated using the formula correlation with $\alpha = 5\%$ and a confidence level = 95%. Large samples are obtained as many as 31 samples for each antibody treatment.

Slide preparation and immuno-histochemistry. Paraffin blocks were taken from patients FKUI based HE preparations that verified by Anatomical Pathology specialists. Paraffin blocks were cut with a thickness of $4\mu\text{m}$ and placed on microscope slides. Slides were dried in an oven at a temperature of $60\text{ }^\circ\text{C}$ for 30 minutes.

Immuno-histochemistry smear using Streptavidin-Biotin complex labeling methods. Primary antibodies were used to label ZIP1 is primary antibody rabbit polyclonal anti-human ZIP1 (Abcam technologies) dilution 1 : 2000, while for Caspase-3 used primary antibody mouse monoclonal anti-human Caspase-3 (Santa Cruz)

dilution 1 : 1600. Included negative control for each cases and a positive control for each batch of staining. Assessment ZIP1 expression and Caspase 3 conducted on 500 tumor cells from 5 different visual fields (magnification $400\times$) were selected randomly. Each represents the field of view of 100 tumor cells. Protein expression tested positive (+) if the chocolate stained on the cytoplasmic membrane and cytoplasm of tumor cells to antibody ZIP1 and chocolate stained on the cytoplasmic Caspase-3 antibodies. Stained cell count performed using ImageJ program (Figure 1 & Figure 2).

Data analysis using SPSS software version 11.5 are descriptive statistics, testing normality using the Shapiro-Wilk test. Comparison of expression profiles ZIP1 and Caspase-3 prostate cancer tissue in a variety of Gleason score is used Kruskal-Walis test or Analysis of Variants (ANOVA). The correlation between the expression of Caspase-3 ZIP1 and prostate cancer tissue at different Gleason scores with Pearson correlation test or Spearman alternative test. Furthermore, the data will be presented in the form of narratives, images, and tables.

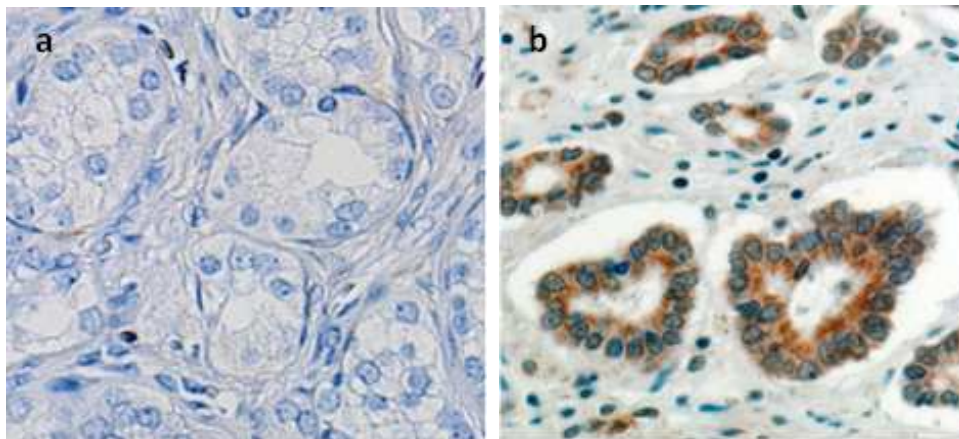


Figure 1. Expression of Negative and Positive Immuno-Histochemical Staining in Prostate Adenocarcinoma (Magnification 400x). (a) Negative Expression (b) The Expression of Caspase-3 Positive

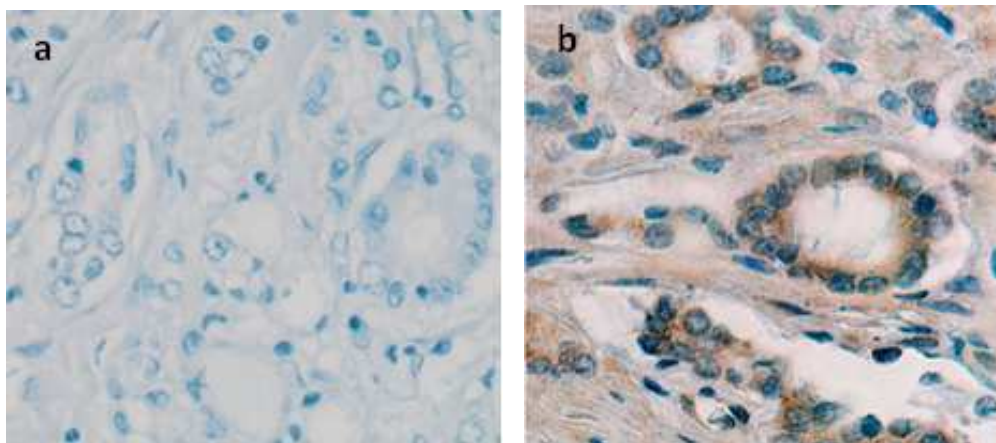


Figure 1. Expression of Negative and Positive Immuno-Histochemical Staining in Prostate Adenocarcinoma (Magnification 400x). (a) Negative Expression (b) Expression Positive ZIP1

Results

This study used 31 cases were confirmed as prostate adenocarcinoma that comes from prostatectomy outcomes preparation and meets the inclusion criteria Immunohistochemical examination of the 31 cases studied were grouped by system Gleason Score (GS) to obtain cases of adenocarcinoma with GS6 counted 4 cases, GS7 counted 9 cases, GS8 counted 3 cases, GS9 counted 11 cases, and GS10 counted 4 cases.

Table 1 shows the expression of prostate adenocarcinoma ZIP1 on the level of Gleason score (GS) significant relationship ($p = 0.08$). While the expression of Caspase-3 in prostate adenocarcinoma with different GS was found significantly different ($p = 0.841$).

In the biomolecular mechanisms, ZIP1 protein acts as a zinc transporter. Allegedly the increase in protein expression ZIP1 will increase the expression of Caspase-3 protein. To see the relationship between the protein and Caspase-3 ZIP1 done analysis Pearson correlation. Results correlation ZIP1 expression and

Caspase-3 in prostate adenocarcinoma can be seen in Figure 3.

This analysis found a significant positive correlation between protein expression ZIP1 and Caspase-3 ($r = 0.379$ and $p = 0.018$). The higher expression significantly ZIP1 followed by increased expression of Caspase-3. To see ZIP1 and Caspase-3 protein expression in prostate adenocarcinoma group of intermediate grade (GS 6-7) and high grade (GS 8-10) performed women different analyzes; the results are shown in Figure 4. The results of the statistical calculation obtained analysis results of the mean ZIP1 protein expression in intermediate-grade and high-grade prostate adenocarcinoma did not differ significantly ($p = 0.76$), as did the mean Caspase-3 protein expression in prostate adenocarcinoma in the intermediate grade and high grade did not differ significantly ($p = 0.15$).

Further, analysis of the correlation between protein expression ZIP1 and Caspase-3 protein on Gleason Score intermediate grade and high grade shows the results as shown in Figure 5.

Table 1. Expression of ZIP1 and Caspase-3 Protein per Group Gleason Score based on the Mean Number of Smear-positive (+) Cells in Prostate Adenocarcinoma

Prostate adenocarcinoma (Grade GS)	Number of Case (n)	Marker Expression	
		ZIP1	Caspase-3
GS 6	4	32.25±2.52	30.50±2.30
GS 7	9	28.11±1.87	36.89±2.16
GS 8	3	17.00±6.24	23.33±2.15
GS 9	11	49.27±2.36	33.91±1.58
GS 10	4	31.75±7.13	37.00±1.22
mean		35.55±2.18	33.71±1.80
<i>p</i>		Kruskal walis test = 0.08	ANOVA test = 0.841

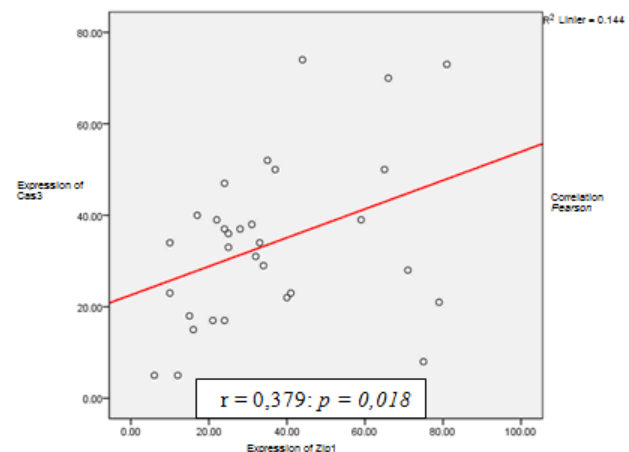


Figure 3. Graph Correlation ZIP1 Protein and Caspase-3 Protein in Prostate Adenocarcinoma

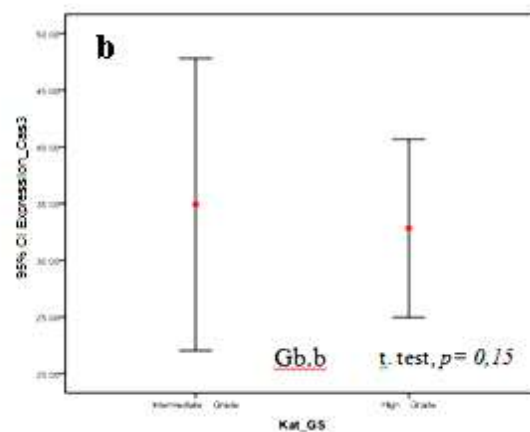
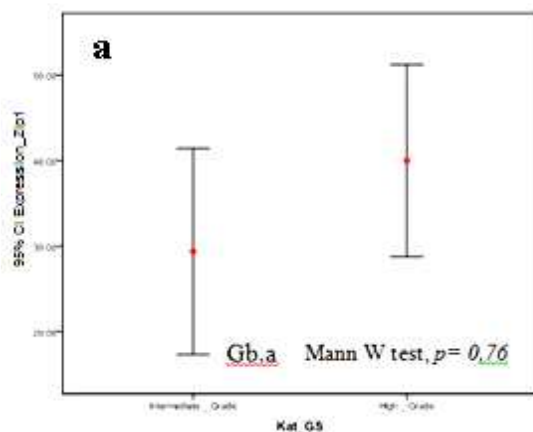


Figure 4. Comparison of ZIP1 and Caspase-3 Mean Protein Expression in the Preparation of Prostate Adenocarcinoma

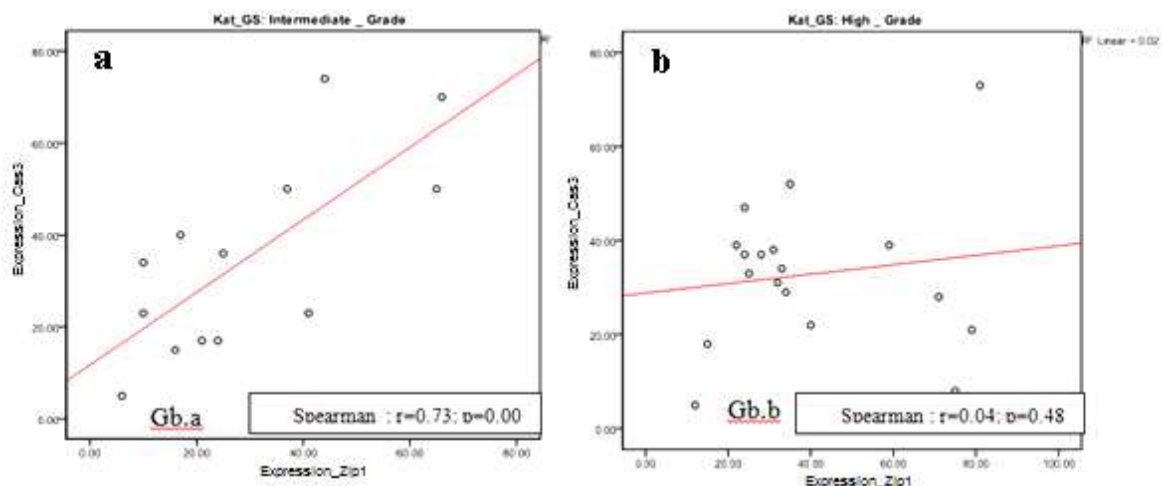


Figure 5. Graph Correlation ZIP1 Protein and Caspase-3 Protein in Prostate Adenocarcinoma. (a) the Average ZIP1 Correlation with Caspase-3 in the Intermediate Grade (b) the Average Correlation ZIP1 with Caspase-3 in High Grade (a) The Mean of ZIP1 Expression in Intermediate Grade and High Grade (b) The Mean of Caspase-3 in the Intermediate Grade and High Grade

There is a significant positive interaction ($r=0.73$, $p=0.002$) between ZIP1 protein expression with Caspase-3 protein expression in the group of intermediate grade (GS 6-7), increasing the ZIP1 protein expression significantly will be followed by increased the expression of Caspase-3 and in other side, the group of high-grade (GS 8-10) the relationship between the expression of ZIP1 protein with Caspase-3 protein expression is very weak and not significant ($r=0.04$, $p=0.48$).

Discussion

Studies of cytotoxic or tumor suppressor effects of zinc in support of a potential zinc treatment approach for prostate cancer is scattered among many published reports. Malignant prostate zinc levels are markedly decreased in all cases of prostate cancer compared to normal/benign prostate. ZIP1 zinc transporter down regulation decreases zinc to prevent its cytotoxic effects. Thus, prostate cancer is a “ZIP1-deficient” malignancy.⁵ ZIP1 protein known to be expressed in several carcinomas such as prostate and breast carcinoma. In this study, the positivity ZIP1 on prostate cancer are lower than in normal prostate. This is in line with research Franklin *et al.* which implies positivity ZIP1 in prostate cancer by 32% and 75% of normal prostate.⁶ ZIP1 protein expression was lower in prostate cancer is thought to cause lower levels of zinc in prostate carcinoma. This is because ZIP1 is for the zinc transporter protein that would cause the loss of the ability of prostate epithelial cells to accumulate zinc and affect the activity of cell proliferation so inhibition of apoptotic cells that can contribute to prostate carcinogenesis.

Decreased ZIP1 expression in prostate adenocarcinoma associated with epigenetic factors that regulate expression of ZIP1 protein.⁶ This is evidenced by research Hlavna *et al.* who stated there was no significant difference in mRNA expression between normal prostate cells with prostate cancer.⁷ Decreased protein ZIP1 allegedly because epigenetic factors, that is deregulation of the post-transcriptional stage that causes the ZIP1 mRNA not translated into functional proteins.

The epigenetic factors are micro RNA (miRNA) that involved in the inhibition of ZIP1 protein synthesis. One of miRNAs that inhibits the ZIP1 expression is miRNA224. This gene is known to elevate in some human prostate cancer cell lines. Also, it also inhibits the expression of micro-RNA metallothionein (MT), the zinc-binding protein that acts as a buffer and donor zinc on intracellular processes.⁷

In this research is also known a decrease in the expression of Caspase-3 in prostate adenocarcinoma were suspected because of the post-translational change break down pro Caspase-3 into Caspase-3. Modification of proteolytic breakdown of precursor form scan causes low levels of activated Caspase-3.⁸ Estrow *et al.* states that the low levels of Caspase-3 indicate a poor prognosis is because most of Caspase-3, undivided and unactivated.⁹ It is thought to be a mechanism of inhibition of apoptosis that supports the development of cancer.

Figure 1 shows a significant positive correlation between ZIP1 and Caspase-3 protein expression. This positive relationship can be explained by the possibility of changes in protein expression ZIP1 which has the lead roles an intracellular zinc transporter, causing changes

in the levels of zinc in cells that trigger the apoptotic signaling cascade, including changes in the activation of Caspase-3. Decreased levels of zinc and citrate occurred starting from the beginning of malignancy.¹⁰ Low Zinc levels can also inhibit apoptosis through the intrinsic cell death pathway. It is characterized by a decrease in mitochondrial membrane potential, followed by caspase activation.³ Caspase-3 are present in the active form can be inhibited by zinc because activation of catalytic caspase motif consisting of cysteine and histidine residues can be bound to zinc on one or both residues the amino acids.¹¹

Further, analysis examine the correlation ZIP1 expression and activity of Caspase-3 in different Gleason score (Figure 5), showed that there was the positive correlation in prostate adenocarcinoma group GS6, 7, and 8. In the GS9 and 10, the relationship is getting weaker. According to Franklin *et al.*¹² grow than a development of prostate malignancies caused by the loss of the ability of prostate cells to accumulate zinc by decreased expression of ZIP1 protein. Decreased zinc plays a role in the inhibition of the formation of membrane pores Bax combined with a decrease in the recruitment of cytosolic Bax, resulting in yeast cytochrome-c is released from mitochondria thus be reduce apoptotic signaling cascade including decrease in Caspase-3.¹⁰

In this study, also did an analysis of the correlation between ZIP1 and Caspase-3 expression based Gleason score grading that is intermediate grade (GS 6 and 7) and a group of high grade (GS 8-10). The results of the analysis (Figure 5a) shows a significant positive correlation ($r = 0.73$) with linear $R^2 = 0.544$ in the group of intermediate grade. This means that from the correlation, 54.4% activation of Caspase-3 is determined by the expression ZIP1, and other factors determine the balance. The reverse at the high-grade group, the correlation is very weak and not significant. Changes in the expression mechanism are made possible by the effect of a decrease in zinc levels that have occurred since the beginning of the development of cancer. Zinc act as a part of the DNA-binding proteins is known as zinc finger proteins that act as transcription factors that bind to short DNA sequences and control the transcription of various genes.¹³ Decreased levels of zinc resulted in the loss of the metal the zinc finger protein.

The loss of zinc in the protein molecule can be replaced by other metals such as cobalt, cadmium, nickel, and iron. Replacement of zinc metal zinc finger protein binds to become one of the causes of carcinogenesis and DNA damage in cells. Cobalt and iron can cause the formation of H_2O_2 , which catalyzes the degradation of deoxyribose and induces DNA damage. So that decreased levels of zinc in prostate adenocarcinoma followed by molecular replacement zinc other metals are expected to

form free radicals that cause damage to DNA (DNA mutation).¹³ The damage (mutations) in the DNA that occurs adenocarcinoma due to reduced levels of zinc (ZIP1 reduction effect) thought to have an impact on the level of malignancy of prostate adenocarcinoma. High-grade GS group had a higher aggressiveness than the GS group of intermediate grade. Higher cell aggressiveness caused DNA mutations that occur in the cell indicated by the activation of Caspase-3 are no longer affected by the concentration of zinc in cells.

Despite decades of research, no efficacious chemotherapy exists for the treatment of prostate cancer. A zinc ionophore (e.g. Clioquinol) treatment to increase malignant zinc levels is a plausible treatment of prostate cancer.⁵ This research can be useful in supporting the determination of the dose of zinc ionophore treatment.

Conclusions

It was concluded that ZIP1 protein expression and Caspase-3 was not correlated with the Gleason Score, but it is also known is a positive relationship between the expression of Caspase-3 and ZIP1 with the adenocarcinoma of the prostate.

Conflicts of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

1. Globocan. Estimated cancer incidence, Mortality, Prevalence, and Disability-adjusted life years (DALYs) worldwide in 2012. (internet) [cited: 2013 December 23] Available from: <http://globocan.iarc.fr>.
2. Sudoyo AW, Setiyohadi B, Alwi I, Simadibrata KM, Setiati S. Buku Ajar Ilmu Penyakit Dalam Ed IV.2006.
3. Franz MC, Anderle P, Burzle M, Suzuki Y, Freeman MR, Hediger MA, *et al.* Zinc transporters in prostate cancer. *Mol Asp Med.* 2012;34:735-41.
4. Costello LC, Franklin RB. The clinical relevance of the metabolism of prostate cancer; Zinc and tumor suppression: connecting the dots. *Mol Cancer.* 2006;5:17.

5. Costello LC, Franklin RB, Zou J, Naslund MJ. Evidence that Human Prostate Cancer is a ZIP1-Deficient Malignancy that could be Effectively Treated with a Zinc Ionophore (Clioquinol) Approach. *Chemotherapy (Los Angel)*. 2015;4:1.
6. Hlavna M, Raudenska M, Hudcova K, Gumulec J, Sztalmachova M, Tanhauserova V, *et al*. MicroRNAs and zinc metabolism-related gene expression in prostate cancer cell lines treated with zinc(II) ions. *Int Jour Onc*. 2012;41:2237-4.
7. Sulpizi M, Rothlisberger U. Molecular dynamics studies of caspase-3. *Biophys J*. 2003;84:2207-15.
8. Estrow Z, Thall PF, Talpaz M. Caspase-2 and caspase-3 protein level predictors of survival in acute myelogenous leukaemia. *Blood*. 1998;92:3090-7.
9. Johnson LA, Kanak MA, Kajdasy-Balla A, Pestaner JP, Bagasra O. Differential Zinc accumulation and expression of human Zinc transporter 1 (hZIP1) in prostate glands. *Methods*. 2010;52:316-21.
10. Niles BJ. Mechanism of zinc deficiency-induced cell death [dissertation]. University of California. 2000.
11. Franklin RB, Milon B, Feng P, Costello LC. Zinc and zinc transporters in normal prostate and the pathogenesis of prostate cancer. *Front Biosci*. 2005;10:2230-39.
12. Alam S, Kelleher SL. Cellular Mechanisms of Zinc Dysregulation: A Perspective on Zinc Homeostasis as an Etiological Factor in the Development and Progression of Breast cancer. *Nutrients*. 2012;4:875-903.
13. Franklin RB, Feng P, Milon B, Desouki MM, Singh KK, Kajdacsy-Balla A, *et al*. hZIP1 zinc uptake transporter down regulation and zinc depletion in prostate cancer. *Mol Cancer*. 2005;4:32.