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hZIP1 Zinc Transporter Down-Regulation in Prostate Cancer Involves the Over Expression of Ras Responsive Element Binding Protein-1 (RREB-1)

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Abstract

Background—A marked decrease in the level of zinc is a consistent characteristic of prostate cancer; which results from down regulation of ZIP1 zinc transporter. The aim of this study was to determine if RREB-1 transcription is involved in the down regulation of ZIP1 gene expression; and to determine the expression of RREB-1 in benign and cancerous prostate in situ.

Methods—Over expression and siRNA knock down of RREB-1 were used to determine the effect of RREB-1 on hZIP1 abundance in PC-3 cells. Immunohistochemistry with tissue microarrays (TMA) and tissue sections was used to determine the levels of RREB-1 expression in prostate in situ.

Results—Over expression of RREB-1 resulted in a decrease in the abundance of hZIP1 in the plasma membrane of PC-3 cells; whereas siRNA knock down significantly increased hZIP1 expression. Prostate TMAs and tissue sections showed an inverse relationship between RREB-1 and hZIP1 staining.

Conclusions—RREB-1 over expression results in down regulation of hZIP1 and contributes to the loss of *hZIP1* expression and zinc in prostate cancer. This is an early event in prostate carcinogenesis.

Keywords

hZIP1; zinc transporter; RREB-1; prostate cancer

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INTRODUCTION

A hallmark characteristic of prostate cancer is the marked decrease in the level of zinc in adenocarcinomatous glands compared to the high zinc level that exists in normal peripheral zone acini [1,2,3]. This relationship is established by numerous corroborating reports over a period of more than sixty years since first reported by Mawson and Fisher in 1952 [1] (for reviews see [4,5]). This decrease has been verified by reported studies on tissue extracts of normal and cancerous tissues and by studies that involve in situ measurements and visualization of cellular zinc levels. These studies reveal that the peripheral zone (the predominant region where malignancy arises and develops) glandular epithelial cells and also BPH cells are zinc-accumulating cells; whereas the malignant cells have lost the ability to accumulate zinc. Despite this well-established relationship, the mechanisms and factors involved in the loss of zinc in malignancy are not understood and thus the clinical significance of the relationship is not fully appreciated.

The loss of zinc accumulation in malignancy in situ is a metabolic transformation that is necessary for prostate carcinogenesis [6]. Zinc exerts antitumor effects that include metabolic effects [7]; inhibition of growth/proliferation and induction of apoptosis [8,9]; and inhibition of migration and invasion [10]. These adverse effects of zinc must be eliminated before normal prostate epithelial cells can complete their progressive transformation to malignant cells. This is achieved by the silencing of hZIP1 (SLC39A1) gene expression and loss of the functional hZIP1 transporter, which is responsible for the cellular uptake and accumulation of zinc in prostate cells [4,5,11].

Therefore the down-regulation of hZIP1 is the important genetic event responsible for the decrease in the prostate zinc level that occurs in prostate carcinogenesis. The expression of hZIP1 in prostate cancer derived cell lines suggests that hZIP1 down regulation is the result of epigenetic silencing of gene expression. Our previous study [12] provided initial evidence that RREB-1 might be involved in the hZIP1 silencing mechanism. This present study provides additional new evidence for the role of RREB-1 in silencing hZIP1 expression.

METHODS AND MATERIALS

Cells and Cell Culture

PC-3 cells were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 medium with L-Glutamine and Hepes supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin mixture. Cells were plated in 12- well plates and twenty-four hours later they were transfected with 1 ug or 2 ug of pcDNA3.1-Finb/RREB1described in [13] and kindly provided by Dr. Leiter or with the empty pcDNA3.1 vector using the Fugene HD transfection reagent from Roche Applied Science.

Immunohistochemistry (IHC)

Protein expression in serial prostate tissue microarrays (TMA) purchased from Biomax-US, Rockville was determined by immunohistochemistry (IHC). The TMA contained 8 cases of prostate adenocarcinoma and 3 normal prostate tissues in duplicate cores per case. IHC with rabbit polyclonal anti-RREB-1 antibody (Abcam, Cambridge, MA) and hZIP1 chicken

polyclonal antibody [14] was performed using standard protocols. Slides were deparaffinized with heating and incubation in xylene. Antigen retrieval was done by heating slides in Tris EDTA, pH 9.0 at 99°C for 20 min., followed by incubating in 3% hydrogen peroxide for 15 min, and blocked with 5% BlokHen (Abcam) for hZIP1 or 1% BSA for RREB1. Slides were then incubated with 10 ug/ml anti-RREB-1 antibody or anti-hZIP1 antibody at 4°C overnight, followed by incubation with the appropriate HRP- labeled secondary antibodies for 30 minutes. Color was developed by incubating slides with DAB +chromogin for 10 min followed by Dako DAB enhancer for 5 min. For some slides hematoxylin was used as counterstain. The appearance of immuno-positivity for RREB1 of the glandular epithelial cells was used for scoring as we previously described [15,16]; negative, no positive cells; + < 10% positive cells; ++ 10–50% positive cells; +++ > 50% positive cells.

Western Blot

Plasma membranes were prepared from PC-3 cells by the procedure previously described [17]. Whole cell lysates were centrifuged at 10,000 × g for 15 min to pellet mitochondria followed by centrifugation at 110,000 × g for 30 min to collect membranes. The membrane proteins were extracted using RIPA buffer (Upstate Biotech) and the concentration determined using the BioRad protein assay based on the Bradford procedure [18]. Aliquots of the whole cell lysates were collected for determining whole cell expression changes. Proteins were separated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes and incubated overnight with hZIP1 antibody. The membranes were stripped of antibody and reprobed using different antibodies for loading controls (ICN Biomedicals).

RESULTS

RREB-1 effects on ZIP1 expression

We had previously reported that the hZIP1 promoter contains a strong inhibitory cis-acting element to which RREB-1 transcription factor binds and represses reporter activity [12]. We now report on the effect of RREB-1 on the expression of hZIP1 in prostate cells. We determined the effect of RREB-1 over expression on the level of hZIP1 in PC-3 cells. Cells were transfected with either empty vector or expression plasmid containing Finb/RREB-1 cDNA. As shown in Fig.1a, transfection resulted in dose dependent increase in expression of RREB-1 in PC-3 cells. Figure 1b shows that the over expression of RREB-1 resulted in a 50 % decrease (Fig.1d) in the level of hZIP1 associated with the plasma membrane. Since the level of hZIP1 in the plasma membrane represents the functionally important zinc uptake transporter, it was important to determine the level in this fraction. However, if transcription regulation is the mechanism of hZIP1 silencing, the level of hZIP1 should also be decreased in the whole cell lysate. Figure 1c shows that over expression of RREB-1 also decreased the level of hZIP1 in the whole cell which is consistent with transcription repression.

We then determined the effect of RREB-1 knock-down on hZIP1 level. We showed previously that RREB-1 knock-down by RNAi increased the transcriptional activity of the hZIP1 promoter in a luciferase assay [12]. Figure 2a shows that transfection with the RREB-1 siRNA resulted in a 100% (Fig. 2c) increase in hZIP1 level compared to non-

transfected and transfection with non-target siRNA. Figure 2b shows that the level of RREB-1 following treatment with RREB-1 siRNA was significantly decreased compared to non-target siRNA. These results are consistent with RREB-1's role in the repression of hZIP1 expression.

RREB1 and hZIP1 expression in prostate tissue samples

These results coupled with previous results [12] now establish that increased RREB-1 represses hZIP1 gene expression and decreased plasma membrane abundance of ZIP1 transporter in prostate cells. It was then important to determine if REBB-1 is increased and hZIP1 is decreased in situ in prostate cancer vs. normal prostate tissue sections. For this study we employed tissue microarray slides that contained prostate cancer cores and normal prostate cores. RREB-1 IHC was performed on one TMA slide and ZIP1 IHC was performed on a serial TMA slide. The advantage of TMA slides is that multiple cancer and normal tissue cores are stained under identical conditions so any visualized differences observed in the cores are not due to technical variations. Also serial TMA slides provide cores from serial sections for comparison of different factors.

Figure 3a shows a representative result from TMA, which reveals that RREB-1 expression is markedly increased in prostate cancer while hZIP1 expression is decreased. Table 1 is a summary of demography, grade, Gleason score, TNM stage and RREB-1 expression analysis for eight cases of histologically confirmed prostate cancer and three normal cores. The results show that all cancer cases were positive (++/+++) for RREB-1 expression while the normal cores were either negative or only slightly positive (−/+). Statistical analysis (unpaired t-test) resulted in a highly significant difference ($P<0.0001$) for these results. In addition, RREB-1 IHC of archived tissue section also shows that RREB-1 is down regulated in adenocarcinomatous glands as compared to adjacent benign glandular epithelium (Fig. 3b). It is also most important to note that the increase in RREB-1 is evident in early stage malignancy (Grade 1, well-differentiated) and persists in advancing malignancy (Grade 2, moderately-differentiated; Grade 3, poorly-differentiated). Thus, the increase in RREB-1 is an early event in the development of malignancy, as is the decrease in zinc and hZIP1 [19,16,11].

DISCUSSION

The in situ RT-PCR ZIP1 expression and the IHC hZIP1 transporter abundance have established that hZIP1 is down regulated in malignant cells in prostate cancer [16,11,20]. Notably, cell lines derived from malignant prostate express hZIP1 at the mRNA and protein levels [14,21,22]. Consequently, the loss of expression in in situ prostate cancer is not due to deletion, permanent genetic mutation, or alteration in the hZIP1 gene; but more likely must result from epigenetic silencing.

Our previous studies showed that one mechanism for decreased hZIP1 expression is down regulation at the transcriptional level [12]. The hZIP1 promoter contains a cis-acting element that inhibits transcriptional activity of the basal promoter. This cis- inhibitory element contains the consensus binding sequence for RREB-1 [12]. Thus the silencing of hZIP1 expression in prostate cancer appears to be due to up-regulation of the RREB-1 transcription

factor which represses expression of the transporter. RREB-1 is a zinc finger transcription factor that is reported to either increase or to repress the expression of target genes depending on the context of the gene promoter and co-factors associated with its binding [13,23]. The results of this study and our previous report on the presence of RREB-1 binding sites in the hZIP1 gene promoter region [12] establish that RREB-1 is a repressor of hZIP1 gene expression. We used PC-3 cells for these studies because, while they are a prostate cancer derived cell line, they express hZIP1 and thus exemplify the epigenetic nature of hZIP1 silencing in prostate cancer. In previous studies we showed using this cell line that the hZIP1 promoter contains a cis-acting repressor element, here we show that under conditions of RREB-1 over expression the level of hZIP1 is decreased. The corresponding results showing RREB-1 up regulation concurrent with hZIP1 down regulation in situ in prostate cancer provides compelling evidence that this is an important gene silencing mechanism.

The loss of zinc in prostate cancer is a consistent and universal finding [24,6]. Even more intriguing is the observation that neighboring areas to cancer that appear histologically normal also exhibit a loss in zinc [19,11]. In addition, ZIP1 down regulation and loss of zinc are shown to occur in PIN [16], which is considered to be a premalignant lesion. These early changes indicate that the loss of zinc accumulation is a necessary and early event in the development and progression of prostate cancer.

RREB-1 is a downstream effector of the Ras-Raf-MEK-ERK pathway [25,26,23]. Previously we showed that transfection of PC-3 cells with constitutively active Ras inhibited hZIP1 promoter activity, while transfection with dominant negative Ras increased promoter activity. These effects were mediated by RREB-1 since they were attenuated when the RREB-1 binding site in the hZIP1 promoter was mutated [12]. The Ras pathway has been shown to be up regulated in prostate cancer [27,28,29]; and interestingly, zinc is reported to be a negative regulator of this pathway [30]. Physiological concentrations of cytosolic zinc inhibit Ras signaling and also inhibit Raf-1 activation downstream of Ras [31]. Thus the activation of the Ras pathway is followed by increased expression of RREB-1 with resultant repression of hZIP1 expression. The loss of hZIP1 protein in the plasma membrane causes the loss of zinc accumulation and would support the continued activation of the Ras pathway. The loss of zinc through this mechanism may be an essential component of the progression of the prostate carcinogenesis process. Clearly further studies of the role of zinc in modulating the Ras-Raf-MEK-ERK pathway are necessary to establish the role of hZIP1 and zinc in prostate cancer development and progression.

It is now evident that the following are concurrent changes that occur early in the development of prostate malignancy: 1) REBB-1 up-regulation (this study); 2) down-regulation of hZIP1gene expression and loss of ZIP1 transporter [16,11] and 3) depletion of zinc [19,16,11]. In addition, the down-regulation of ZIP1 and the depletion of zinc have been identified in PIN and in non-malignant loci neighboring malignant cells [19,16,11]. Moreover, zinc has been shown to exhibit tumor suppressor effects in malignant prostate cells in in vitro and in vivo studies (reviewed in [4]). Additionally citrate decrease in malignancy which is due to decreased zinc is an early event [32] (many MRS studies reviewed in [33]) which parallels the decrease in zinc in prostate cancer.

These concurrent events provide the clinical and experimental data in support of our concept of the role of zinc in prostate carcinogenesis. Carcinogenesis is still a poorly understood oncological process and subject to varying concepts. We initially proposed [6] a genetic/metabolic role of zinc in prostate carcinogenesis. Additional information since then supports the concept and leads to our current concept that is presented in figure 4. The concept is based on the view that carcinogenesis is a multi-step process that involves three phases: the initiating genetic neoplastic transformation; followed by the genetic/metabolic facilitative phase; which leads to the malignant phase. The important point in this concept is that the neoplastic cell type is not a malignant cell because it does not possess the metabolic conditions to support the energetic/synthetic/catabolic requirements for the manifestation of malignancy. All cells must have the cellular metabolic requirements to support the activity of the cells: and when the activity of a cell changes, its metabolism must change to support that activity. This applies to the transformation of normal cells to malignant cells.

We apply this concept to the role of hZIP1 and zinc as a genetic/metabolic facilitative event in the transformation of the neoplastic cell to the malignant cell as represented in figure 4. The new information regarding the involvement of RREB-1 is associated with this transformation as an important genetic event for the silencing of hZIP1 as shown in Figure 4. This event occurs in the transformation of the neoplastic cell to a premalignant cell leading to malignancy. It is important that we emphasize that our representation in figure 4 employs the zinc-citrate relationship, and does not exclude the obvious involvement of a multiplicity of many other relationships.

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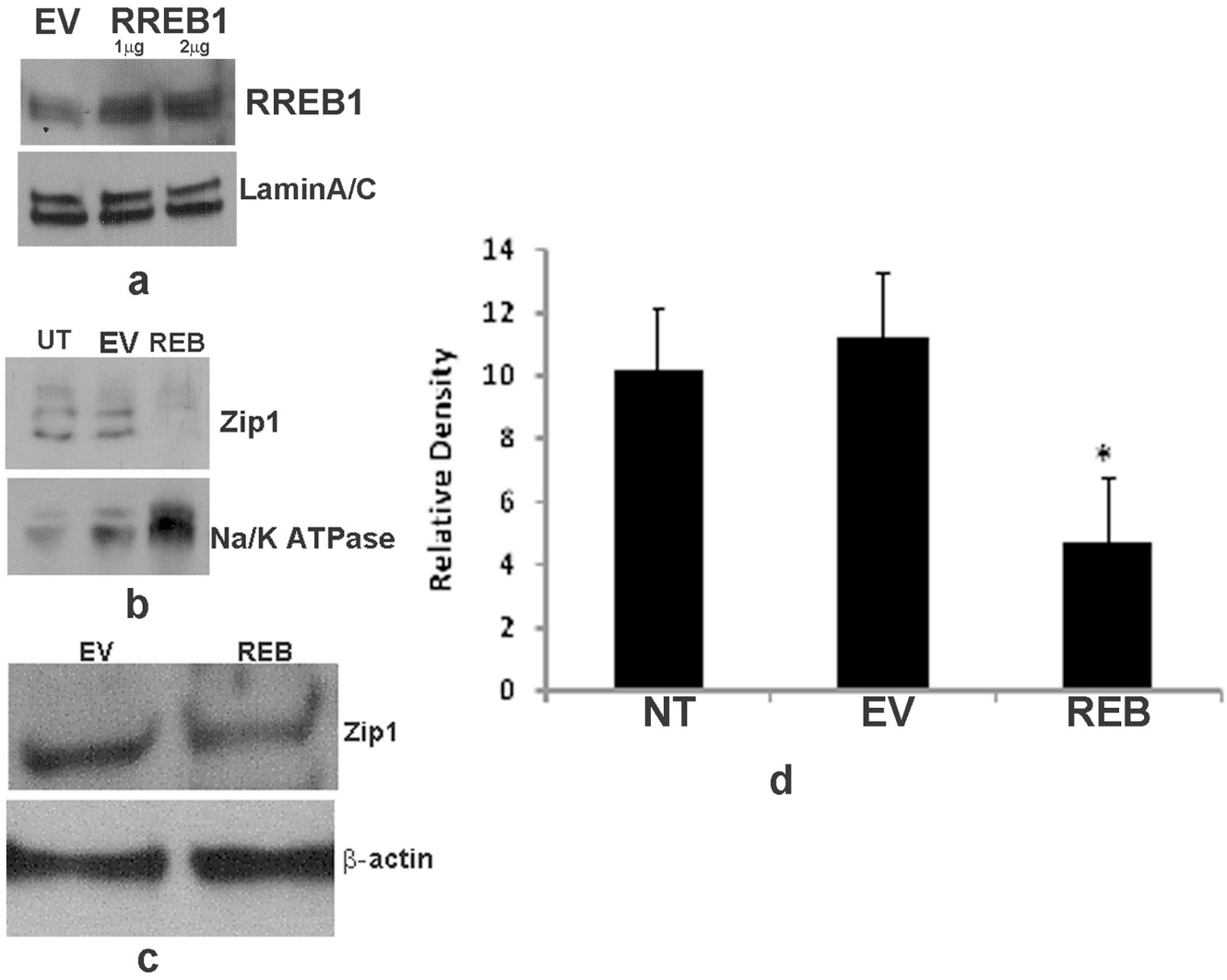

Figure 1.

Effect of RREB-1 over expression on ZIP1 abundance. A) RREB-1 expression in nuclear fraction of PC-3 cells after transfection with 1.0 and 2.0 μg of pcDNA3.1-Finb/RREB-1. Lamin A/C was used as a SDS-PAGE loading control; EV=empty vector. (b) hZIP1 expression in the membrane fraction of PC-3 cells 24 hr after transfection with pcDNA3.1-Finb-RREB-1. Na/K APTase was used as a gel loading control; UT=un-transfected, EV=empty vector; REB=Finb/RREB-1 expression vector. (c) hZIP1 expression in the whole cell lysate of PC-3 cells 24 hr after transfection with pcDNA3.1-Finb-RREB-1. β-actin was used as a gel loading control; EV=empty vector; REB =Finb/RREB-1 expression vector. (d) Quantification of band intensities for ZIP1 using ImageJ software. *=$p<0.05$, N=3 independent experiments.

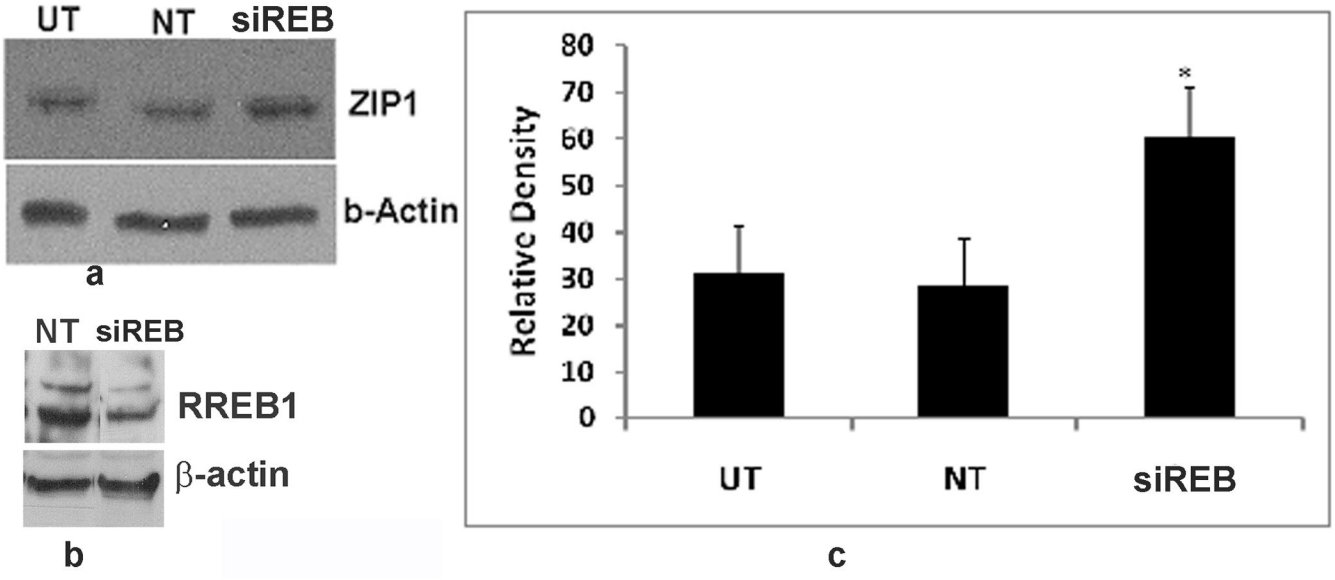

Figure 2.

Effect of RREB-1 knock-down on hZIP1 expression. (a) hZIP1 expression in membrane fraction of PC-3 cells after transfection with RREB-1 siRNA. (b) RREB-1 level in whole cell lysate after transfection with RREB-1 siRNA. β-actin was used as a gel loading control. UT =untransfected; NT= non-target siRNA; siREB=RREB-1 siRNA. (c) Quantification of band intensities for hZIP1 in membrane fraction using ImageJ software; *=P<0.05, N=3.

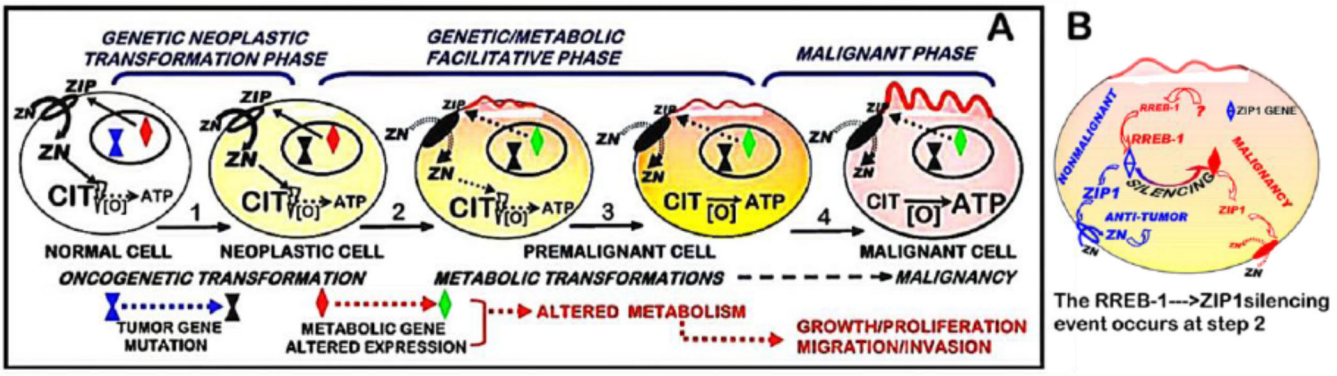

Figure 3.

Immunohistochemistry staining of prostate biopsy cores from tissue microarrays. (a) TMA shows high abundance of hZIP1 in normal compared to prostate cancer. Conversely, RREB-1 staining shows high abundance in prostate cancer compared to normal prostate. (b) Prostate tissue section showing high abundance of RREB-1 in cancer area compared to low abundance in normal/BPH area. Conversely high abundance of hZIP1 shown in normal/BPH areas compared to carcinoma areas. Blue arrows indicate plasma membrane location of hZIP1 in normal/BPH region.

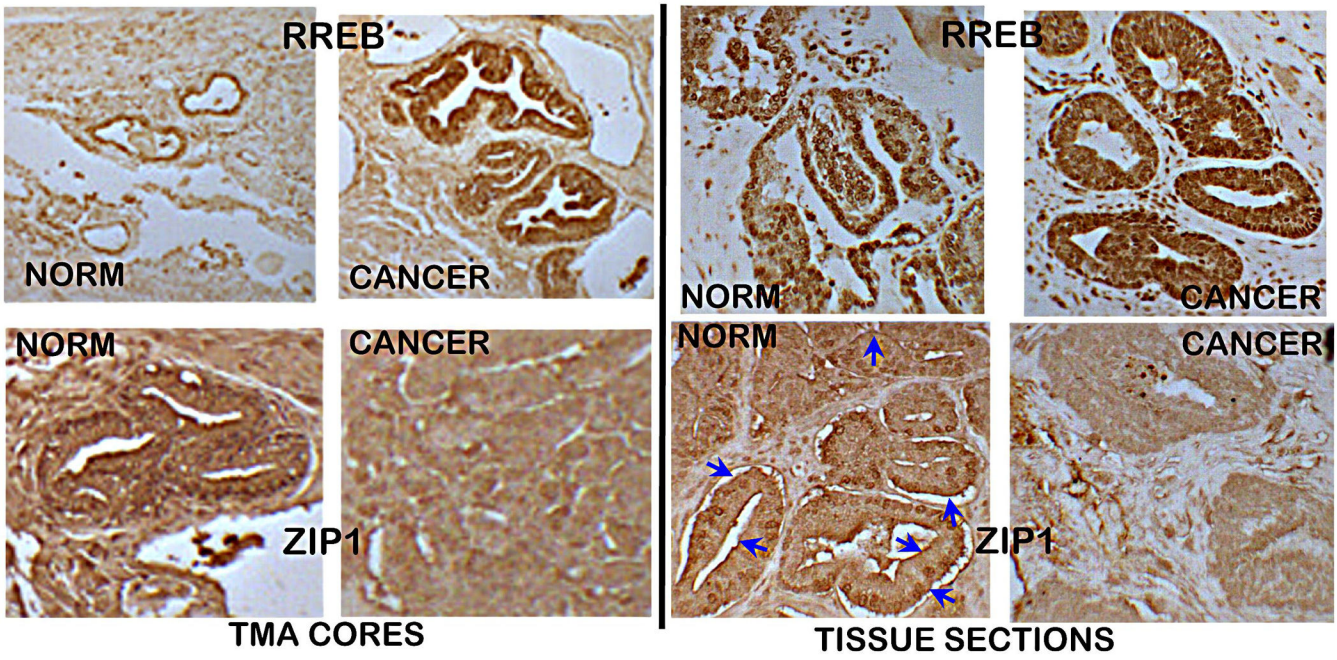

Figure 4.
Concept of prostate carcinogenesis with specific roles of RREB-1, hZIP1 and zinc and citrate.

Table 1

RREB-1 immunohistochemistry, age and histopathologic data of prostate adenocarcinoma (n=8) and normal prostate (n=3) tissue microarray

No	Age	Grade	Gleason Score	TNM	RREB-1 SCORE
1	64	1	1+1	T1N0M0	++
2	70	2	4+3	T3N0M0	+++
3	71	2	3+3	T2N0M0	++
4	64	2	3+4	T2N0M0	+++
5	61	2	3+4	T3N1M0	+++
6	65	3	2+4	T2N0M0	+++
7	66	2	4+3	T3N0M0	++
8	62	3	3+5	T2N0M0	+++
9	73	normal	n/a	n/a	0 (Neg)
10	28	normal	n/a	n/a	+
11	43	normal	n/a	n/a	0 (Neg)

Score means (sem) unpaired t-test PCa=2.75(0.16); Norm=0.33(0.33) Pvalue <0.0001

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