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Research Article

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ZIP11 and ZnT1 are Differentially Expressed in Human Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is a solid, malignant, and heterogeneous tumor originating in the renal tubular epithelium. There are several histopathological classifications, with clear-cell carcinoma (ccRCC) being the most common. Its occurrence is associated with mutations in the von Hippel-Lindau gene, which regulates cellular responses to stress, division, death, and differentiation. The loss of functionality affects the degradation of hypoxia-inducible factor (HIF). In turn, the HIF1α subunit becomes highly expressed. Zinc (Zn) is the second most abundant trace element in the human body, and small variations in its concentration can lead to disturbances in the cellular environment. Zn transporters play a fundamental role in Zn homeostasis. In this study, the expression and localization of the Zn channels ZIP11 and ZnT1 were verified in normal tubular renal cells (HK-2) and a clear cell renal adenocarcinoma line (786-0). RT-PCR and western blotting data showed that the ZIP-11 channel was highly expressed in tumor cells. In contrast, the ZnT1 channel was expressed at lower levels in tumor cells. Immunofluorescence data revealed different localization patterns between the two lines. The expression of ZIP-11 in HK-2 cells was predominantly cytoplasmic, while in 786-0 tumor cells, in addition to strong cytoplasmic staining, nuclear staining was also observed. ZnT1 expression in HK-2 cells was cytoplasmic and accentuated on the cell membrane, while that in 786-0 tumor cells was nuclear. These results demonstrated different expression patterns of the key Zn transporters, ZIP11 and ZnT1, in RCC cells compared to those in the normal kidney epithelium. Further elucidation of the molecular mechanisms underlying Zn dysregulation in RCC may reveal potential therapeutic targets.

Keywords: Renal cell adenocarcinoma, Gene expression, Zinc transporters

Introduction

Renal cell carcinoma (RCC) is one of the most common malignancies, accounting for over 90% of all kidney cancer cases [1]. The most common subtypes of RCC are clear cell RCC (ccRCC), papillary RCC, and chromophobe RCC, accounting for 75%–80%, 10%–15%, and 5% of all RCCs, respectively [2]. ccRCC arises from the malignant transformation of renal tubular epithelial cells, and the most frequent mutation is in the Von Hippel Lindau (VHL) tumor suppressor gene [3]. Proteins encoded by the VHL gene can negatively regulate a series of intracellular proteins, including hypoxia-inducible factor (HIF). Mutations in the VHL gene lead to the dysfunction of VHL proteins. Without functional VHL, HIF is not targeted for degradation, leading to its accumulation in cells. Elevated HIF levels promote the expression of angiogenic factors like vascular endothelial growth factor [4].

Zinc (Zn) is an essential trace element that plays a vital role in various cellular functions. Within the cells, Zn is found both in a protein-bound state and as a free or chelatable divalent ion. In its protein-bound form, Zn has a structural function and is essential for the activity of various cytosolic and nuclear enzymes [5]. More than 300 enzymes and approximately 2000 transcription factors require Zn to maintain their structural integrity and DNA-binding activity [6-8]. Free Zn ions, often referred to as labile Zn, migrate between cellular compartments and bind to numerous proteins, thereby altering their biological activities [9].

The control of cellular the Zn homeostasis is carried out by Zn importers, Zrt- and Irt-like proteins (ZIP), Zn transporters (ZnT), and metallothioneins (MT) [10]. ZIP and ZnT regulate the transport of Zn through the cellular membrane and between cellular compartments. ZIP allows the uptake of Zn from the extracellular medium or the efflux of intracellular organelles into the cytoplasm, thereby promoting an increase in cytosolic Zn levels of Zn. In contrast, ZnT channels transport Zn from cells or into intracellular storage compartments, thereby reducing cytosolic Zn concentrations [11].

Metallothioneins (MTs) are a family of small, cysteine-rich proteins that play crucial roles in regulating Zn bioavailability and homeostasis. MTs sequester, mobilize, and release Zn ions in response to various cellular stimuli and environmental conditions [12].

The dysregulation of Zn homeostasis is associated with various diseases, including neurodegenerative disorders, growth retardation, cardiac diseases, and cancer [13-16]. A review by Sugimoto et al. showed that serum Zn levels decreased in patients with tumors, including esophageal, breast, lung, liver, prostate, pancreatic, and gynecological tumors, compared to those in healthy individuals [16]. However, data regarding the role of Zn in RCC are scarce. In vitro studies have shown that treatment with low concentrations of Zn chloride inhibits the proliferation, migration, and invasion of ccRCC cells. Yu et al. demonstrated that Zn treatment of human renal carcinoma (A498) cells caused a significant increase in autophagy [17]. In contrast, Wang et al. found a positive association between dietary Zn and the risk of kidney cancer in an epidemiological study of 229 patients with ccRCC, suggesting that this element is implicated in renal carcinogenesis [18]. The aim of the current study was to evaluate and compare the expression of ZIP 11 and ZnT1 Zn channels in normal kidney epithelium and human renal cell carcinoma cell lines.

Materials and Methods

Cell Lines and Culture Conditions

The human clear cell renal adenocarcinoma with VHL gene mutation, 786-0, was purchased from the American Type Culture Collection (ATCC® CRL-1932; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (all obtained from Gibco, Waltham, MA, USA).

The renal proximal tubule epithelial cells, HK-2, were purchased from ATCC (ATCC® CRL- 2190). Cells were cultured in DEMEM + F12 medium in 3/1 proportion respectively, supplemented with 10% FBS (Life Technologies), 300 µg/mL streptomycin and 100 U/mL of penicillin (Life Technologies).

Cells were maintained in a humid chamber at 37°C with 5% CO_2. Triplicate samples of each strain were used for all techniques.

RNA Extraction and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was then synthesized using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol using 2 µg of RNA and was stored at −20°C. The Absolute SYBR Green qPCR Mix® (Invitrogen, Carlsbad, CA, USA) was used for qRT-PCR according to the manufacturer's instructions. Reactions were carried out in 10 µL and under the following PCR conditions: 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 h, and elongation at 72°C for 1 min. Target gene expression levels were normalized to the transferrin receptor (TFRC) mRNA levels. The primers used were as follows: ZIP11, 5′-CCTTGGAGCGGCTTTTGTC-3′ (forward) and 5′-CTTCTGCTGCACCCAAGTGA-3′ (reverse); ZnT1, 5′-GCCTTGGGTTCAGTGATTGTAGT-3′ (forward) and 5′-CCCCTTCAGAACAACCTTTCC-3′ (reverse); TFRC, 5′-GGAGGACGCGCTAGTGTTCT-3′ (forward) and 5′-TGCTGATCTAGCTTGATCCATCA-3′ (reverse). Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. PCR was performed using the ABI Prism 7000 quantitative PCR system (Applied Biosystems, Foster City, CA, USA).

Protein Extraction and Western Botting Analysis

Proteins were extracted from cells using CelLytic™ (Sigma-Aldrich). After centrifugation at 20.000 × g at 4ºC for 15 min, the supernatant was collected and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added.

Protein concentration was determined using the bicinchoninic acid method, and protein samples were stored at −80°C until use. Proteins (40 µg per lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinyl difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA), which were then blocked in 5% skim milk in Tris-buffered saline at room temperature for 1 h. Membranes were then incubated overnight at 4°C with the primary antibodies (all diluted 1:500): anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-365062, monoclonal, mouse-Santa Cruz Biotechnology, Dallas, TX, USA) and Anti-ZIP11 (MBS9005379, policlonal, rabbit-My-Biosource, San Diego, CA, USA). The membranes were then incubated with goat anti-rabbit (ZnT1 antibody: Anti-ZnT1 (MBS9606086, policlonal, rabbit-My-Biosource, San Diego, CA, USA) or goat anti-mouse (62-6420, Invitrogen) horseradish peroxidase-conjugated secondary antibodies (diluted 1:1000) for 2 h at room temperature. Immunoreactive protein bands were detected using a SuperSignal® West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific). Images were acquired using the Uvitec Cambridge Alliance 4.7 (Uvitec Cambridge, Cambridge, UK).

Immunofluorescence Analysis

The isolated cells were plated in 24-well plates and fixed in 4% paraformaldehyde at room temperature for 10 min. The cells were then washed with PBS and incubated with 1% BSA at 37ºC for 1 h to block non-specific interactions. The primary antibody against Anti-ZIP11 (MBS9005379, policlonal, rabbit; My-Biosource), and Anti-α-Tubulin (sc-8035, monoclonal, mouse; Santa Cruz Biotechnology) were incubated with an anti-IgG secondary antibody conjugated with Alexa Fluor 645 (Invitrogen) at room temperature for 1.5 hours. Subsequently, the cells were washed with PBS and maintained in mounting media (Prolong Gold Antifade Reagent, Invitrogen) containing 40, 6-diamidino-2-phenylindole (DAPI) in the darkroom at 4ºC until acquisition of images using a premixed solution of 4,6-diamidino-2-phenylindole (DAPI) and antifade mounting media (ProLong Gold antifade reagent with DAPI; Invitrogen). The fluorescence signals were observed under a fluorescence Nikon E-800 microscope (Tokyo, Japan), and

images were obtained with a DXM1200F digital camera (Nikon Instruments Inc., Melville, NY, USA) and analyzed using the EclipseNet1 software for Nikon cameras.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Results are presented as mean ± standard deviation. Single comparisons of the mean values were performed using Student's t-test. Multiple comparisons were performed using a one-way analysis of variance (ANOVA), followed by Bonferroni's test. Statistical significance was set at P<0.05, P<0.01, and P<0.001.

Results

In this study, we performed in vitro experiments using cell lines derived from normal (HK-2) and human tumor tissue (786-0) cells. In all experiments, the cell viability was never below 95% in either cell line.

RT-qPCR revealed that ZIP11 and ZnT1 were differentially expressed in HK-2 (normal) and 786-0 (tumor) cells. On average, ZIP11 mRNA expression in 786-0 cells was nearly 8.0-fold higher than that in HK-2 cells (P<0.01) (Figure 1A). In contrast, ZnT1 expression was 2.5-fold lower in HK-2 cells (Figure 1B).

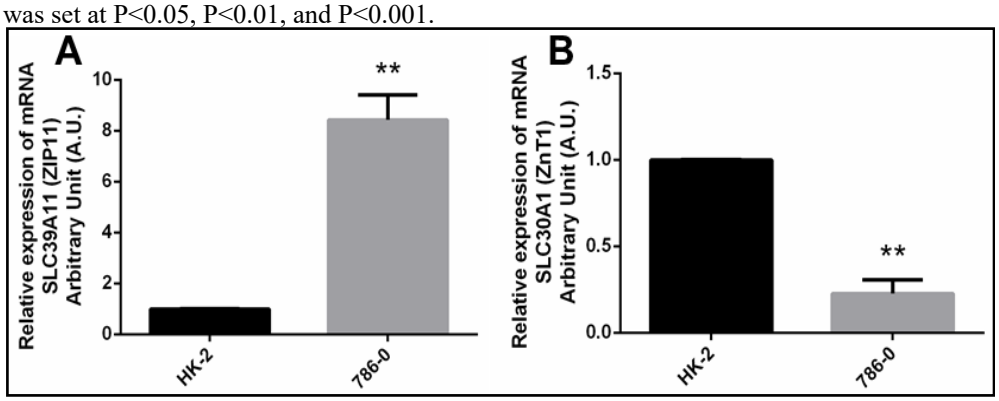

Figure 1: (A) ZIP11 gene expression. (B) ZnT1 gene expression. The gene expression profile was evaluated by qPCR. Values represent mean±SEM (error bars). Student's t-test **p < 0.01. (N=4).

Western blotting was performed for both cell lines as a complementary test to evaluate the ZIP11 and ZnT1 channel expression levels. All the samples showed bands at approximately 35 kDa for ZIP-11 and 55 kDa for–ZnT1 (Figure 2A). Densitometric quantification of the immunoblot band data corroborated the RT-PCR findings. ZIP-11 expression was approximately 35% greater in 786-0 cells than that in HK-2 cells (P<0.05; Figure 2B). In contrast, the concentration of ZnT1 decreased by approximately 50% in 786-0 cells compared to that in HK-2 cells (P<0.05; Figure 2C).

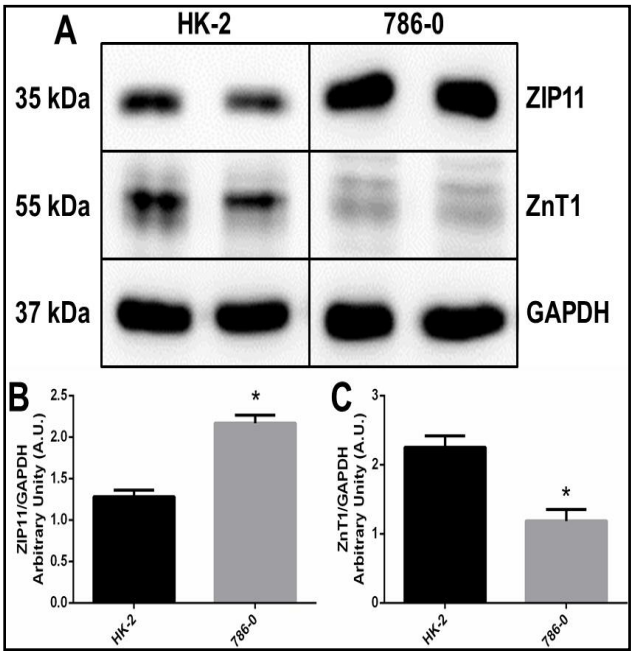

Figure 2: A) Western blot analysis. (B-C) ZIP11 and ZnT1 optical density values of the corresponding bands were quantified using ImageJ software, with GAPDH serving as an internal control. Values represent mean±SEM (error bars). Student's t-test *, p <0.05. (N=3).

Immunofluorescence data revealed different expression patterns and localizations of ZIP-11 and ZnT1 channels in HK-2 and 786-0 cells (Figure 3). The expression of ZIP-11 in HK-2 cells was predominantly cytoplasmic, while in 786-0 tumor cells, nuclear staining was also observed in addition to strong cytoplasmic staining (Figure 3A). The expression of ZnT1 in HK-2 cells was cytoplasmic and accentuated on the cell membrane. In 786-0 tumor cells, nuclear staining was observed (Figure 3B).

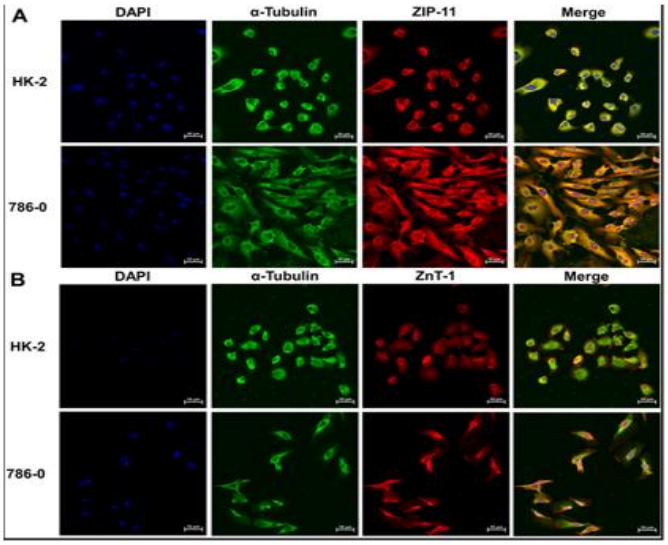

Figure 3: Representattive images of ZIP-11 and ZnT-1 immunofluorescence staining in HK-2 and 786-0cells. A. The immunofluorescence staining of ZIP-11. B The immunofluorescence staining of ZnT-1 and α-Tubulin cells. Original magnification (20X).

Discussion

Renal cell carcinoma (RCC) is a common malignancy, with clear-cell RCC (ccRCC) being the most common subtype. The molecular underpinnings of RCC, particularly the dysregulation of the Von Hippel-Lindau (VHL) gene and the subsequent disruption of the hypoxia-inducible factor (HIF) pathway, have been extensively studied [1,3]. This study aimed to explore the role of Zn homeostasis in RCC.

Zn is an essential micronutrient with multifaceted roles in cellular processes. Its involvement in the regulation of enzymatic activities and transcription factor function underscores its significance in maintaining cellular homeostasis [5,6,7]. Dysregulation of Zn homeostasis has been implicated in various diseases, including cancer [13-16]. A previous study by Sugimoto et al. highlighted decreased serum Zn levels in patients with cancer, suggesting a potential association between Zn deficiency and tumorigenesis [16].

This study focused on investigating the expression patterns of ZIP11 and ZnT1, which are key players in cellular Zn transport in normal kidney epithelium and RCC cell lines. We observed significant upregulation of ZIP11 and downregulation of ZnT1 in RCC cells compared to that in normal kidney epithelial cells. These findings are consistent with those of previous studies that implicated altered Zn transporters in cancer development and progression [17,18].

RT-qPCR, western blotting, and immunofluorescence analyses consistently demonstrated the differential expression and localization of ZIP11 and ZnT1 in RCC cells compared those in to normal kidney cells. These alterations in the expression patterns suggest a potential role for Zn deregulation in RCC pathogenesis. At the cellular level, ZIP11 is proposed to be localized to the nucleus and Golgi apparatus and is thought to regulate nuclear Zn homeostasis, contributing to cell proliferation, among other carcinogenic phenotypes [19]. The role of ZIP11 in the pathophysiology of RCC was demonstrated by Wu et al. The authors demonstrated an association between RCC risk and a ZIP11 variant (rs8081059) [20]. The same ZIP11 expression pattern was observed in Capan-1 pancreatic cell lines, and in pancreatic and breast cancers [19, 21,22].

ZnT1 is the only Zn transporter predominantly located in the plasma membrane where it plays a pivotal role in the export of cytosolic Zn to the extracellular space [23]. ZnT1 is thought to increase intracellular Zn levels. In turn, high Zn levels have been correlated with accumulation of transcriptionally active HIF-1α and increased cellular proliferation [24]. Our immunolocalization data showed that, in HK-2 cells, ZnT1 was the most abundant protein in the cellular membrane. Surprisingly, we found that ZnT1 was localized in the nucleus. ZnT1 is also reported to be localized in the perinuclear region of H4IIE cells, primary hepatocytes, and mouse mammary epithelial cells, indicating that ZnT1 localization is tissue-specific [25]. However, further experiments are necessary to clarify the purpose of this differential localization in 786-0 cells.

In summary, novel insights into the dysregulation of Zn homeostasis in RCC were provided, highlighting the altered expression of key Zn transporters, ZIP11 and ZnT1, in RCC cells compared to that in normal kidney epithelium. Further elucidation of the molecular mechanisms underlying Zn dysregulation in RCC may reveal

potential therapeutic targets.

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