VHL DEPENDENT EXPRESSION OF REDD1 AND PDK3 PROTEINS IN CLEAR-CELL RENAL CELL CARCINOMA

VHL ZAVISNA EKSPRESIJA PROTEINA REDD1 I PDK3 U SVETLOCELJISKOM KARCINOMU BUBREGA

Bojana B Ilic1, Jadranka A Antic1, Jovana Z Bankovic1, Ivana T Milicevic1, Gordana S Rodic1, Dusan S Ilic1, Cane D T ulic2, Vera N Todorovic3, Svetozar S Damjanovic1

1Clinic for Endocrinology, Diabetes and Metabolic Diseases, Medical School, University of Belgrade, Department of Neuroendocrine Tumors and Hereditary Cancer Syndromes, Belgrade, Serbia
2Clinic for Urology, Medical School, University of Belgrade, Belgrade, Serbia
3Institute for Histology and Embryology, School of Medicine of Military Medical Academy, University of Defense, Belgrade, Serbia

Summary

Background: Sporadic clear-cell renal cell carcinoma (ccRCC) is associated with mutations in the VHL gene, upregulated mammalian target of rapamycin (mTOR) activity and glycolytic metabolism. Here, we analyze the effect of VHL mutational status on the expression level of mTOR, elf4E-BP1, AMPK, REDD1, and PDK3 proteins.

Methods: Total proteins were isolated from 21 tumorous samples with biallelic inactivation, 10 with monoallelic inactivation and 6 tumors with a wild-type VHL (wtVHL) gene obtained from patients who underwent total nephrectomy. The expressions of target proteins were assessed using Western blot.

Results: Expressions of mTOR, elf4E-BP1 and AMPK were VHL independent. Tumors with monoallelic inactivation of VHL underexpressed REDD1 in comparison to wtVHL tumors (P = 0.042), tumors with biallelic VHL inactivation (P < 0.005) and control tissue (P = 0.004). Additionally, REDD1 expression was higher in tumors with VHL biallelic inactivation than in control tissue (P = 0.008). Only in wt tumor samples PDK3 was overexpressed in comparison to tumors with biallelic inactivation of VHL gene (P = 0.012) and controls (P = 0.016). In wtVHL ccRCC, multivariate linear regression analysis revealed that 97.4% of variability in PDK3 expression can be explained by variations in AMPK amount.

Address for correspondence:
Svetozar S Damjanovic
Clinic for Endocrinology, Diabetes and Metabolic Diseases, Medical School, University of Belgrade, Department of Neuroendocrine Tumors and Hereditary Cancer Syndromes, Dr Subotica 15, Belgrade, Serbia
Phone: +381113639702; Fax: +38111685357
e-mail: svetadamjanovic@gmail.com

List of abbreviations: C, control renal tissue; M/LOH, VHL-mutated or loss of heterozygosity of 3p locus; M+LOH, VHL-mutated and loss of heterozygosity of 3p locus; WT, wild-type VHL.
Conclusions: Expressions of mTOR, elf4EBP1 and AMPK were VHL independent. We have shown for the first time VHL dependent expression of PDK3 and we provide additional evidence that VHL mutational status affects REDD1 expression in sporadic ccRCC.

Keywords: clear-cell renal cell carcinoma, mammalian target of rapamycin, pyruvate dehydrogenase kinase 3, regulated in development and DNA damage responses, VHL gene

Introduction

The von Hippel-Lindau tumor suppressor gene (VHL) is frequently mutated in sporadic form of clear-cell renal cell carcinoma (ccRCC) (1, 2). Inactivation of the VHL gene contributes to early phases of renal tumorigenesis by accumulation of hypoxia inducible factors (HIFs) and deregulation of processes important for renal epithelial cell morphology (3, 4). Elevated activity of mammalian target of rapamycin (mTORC1/mTORC2) signaling pathways were noticed in different types of tumors including clear-cell renal carcinoma (5, 6). It is well known that mTORC1 controls cellular growth, proliferation and metabolism via its effector molecules p70S6K1 (Ribosomal protein S6 kinase beta-1) and elf4EBP1 (Eukaryotic Translation Initiation Factor 4E Binding Protein 1) and that it is under the control of AMPK (5’ adenosine monophosphate-activated protein kinase) and REDD1 (Regulated in Development and DNA Damage Response 1) (7–15). Additionally, mTORC1 regulates ribosomal biogenesis and nucleolar size (16). The Fuhrman nuclear grading system, an independent prognostic predictor for ccRCC, is based on nucleolar prominence and can be used as an indicator of mTORC1 activity (17). It has been shown that cancer cells utilize glycolysis as the main metabolic pathway for energy production even under normoxic conditions (18–20). The role of these molecules in the regulation of pyruvate dehydrogenase kinases (PDKs) is not well defined in ccRCC. Many studies were focused on the PDK1 isofrom in different carcinomas, but there is no information about the expression level of PDK3 isofrom in ccRCCs (21–23).

In the present study, we evaluated the effect of VHL mutational status on the expression level of mTOR, elf4EBP1, AMPK, REDD1, and PDK3 proteins in ccRCC.

Material and Methods

This study was performed on 37 tumor and corresponding healthy renal tissue samples obtained from patients who underwent nephrectomy due to unilateral kidney tumor without history of hereditary VHL syndrome. All the patients provided written informed consent for participation and the study received the permission from the local Research Ethics Committee. Tissues were sampled after surgery, immediately frozen in liquid nitrogen and stored at –80 °C until use. All tumors were classified as clear-cell renal cell carcinoma by the pathologist.

Protein isolation and Western blot

Proteins were extracted from tumors and corresponding healthy renal tissue. Thirty mg tissue samples were dissected, homogenized and lysed in RIPA buffer containing complete EDTA-free protease inhibitor cocktails (Roche Applied Science, Mannheim, Germany) and left on ice for one hour. Samples were sonicated three times for 30 s followed by cooling for one minute and stored for one hour on ice. Samples were centrifuged for 20 min, 11 000 rpm at 4 °C. Supernatants were pipetted into new tubes. Bio-Rad Protein Assay was used for measurement of protein concentration at 595 nm. Prior to SDS-PAGE separation, proteins suspension was mixed with loading and sample buffer (NuPAGE LDS Sample Buffer, NuPAGE Reducing Agent, Invitrogen Life Technologies, Grand Island, New York, USA), denatured for 10 min at 90 °C and loaded into precast 4–12% Bis-T ris or 3–8% Tris-acetate gels (Invitrogen Life Technologies, Grand Island, New York, USA), depending on the molecular weight of detected proteins. Separated proteins were blotted on nitrocellulose/PVDF membranes using wet electrotransfer devices. Membranes were blocked with 5% nonfat milk in 1XTBST containing 0.1% Tween 20 for one hour at room temperature and incubated in primary antibody in 1XTBST overnight at 4 °C. [anti-mTOR (1:1000, 7C10, Cell Signaling Technology, Danvers, Massachusetts, USA); anti-elf4E-BP1 (1:1000, ab2606, Abcam, Cambridge, UK); anti-AMPK (1:500, ab80039, Abcam, Cambridge, UK); anti-REDD1 (1:500, H-110: sc-67051, Santa Cruz Biotechnology, Dallas, Texas, USA); anti-PDK3 (1:1000, LS-C111083, Lifespan Biosciences, Seattle, Washington, USA); anti-β-actin (1:1000, ab3280, Abcam, Cambridge, UK)] Then, membranes were washed in 1XPBST, probed with appropriate secondary HRP-conjugated anti-mouse or anti-rabbit antibody for 1.5 hours at room temper-
ture and washed in PBST. Proteins were visualized by chemiluminescence (Lumi-light PLUS Western Blotting kit, Roche Applied Science, Mannheim, Germany). After visualization, membranes were stripped with 0.2 mol/L NaOH, and reprobed with another primary antibody or anti-β-actin antibody. The intensities of immunoreactive bands were determined using ImageQuant 5.2 software (GE Healthcare, Little Chalfont, UK). Protein loading was normalized to β-actin (24).

Statistical analysis
All data are presented as mean ± SE. Differences between tumorous and corresponding non-tumorous tissues were estimated using nonparametric Kruskal Wallis analysis of variance followed by Mann Whitney test or ANOVA with Bonferroni correction, depending on data distribution. Pearson's test was used to detect correlation between two variables. Stepwise multivariate analysis was used to determine causal relationships between variables. P value < 0.05 was considered statistically significant. Statistical analyses were done using SPSS® 13.0, Inc., Chicago, Illinois, USA.

Results
Tumor classification based on VHL gene mutational status
We used tumor samples with known mutational status in the VHL gene previously reported by our group (25). Among 37 samples of ccRCCs, in 21 (56.8%) tumors biallelic inactivation of VHL gene was detected (intragenic mutation plus loss of heterozygosity on 3p locus), 10 (27.0%) tumors have shown monoallelic inactivation of VHL (mutation or LOH on 3p locus) and in 6 (16.2%) tumors no alterations in VHL were found (wt VHL). Also, mutations or LOH were not found in all corresponding non-tumorous tissues.

Western Blot analyses: mTOR, eIF4E-BP1, AMPK, REDD1, and PDK3 in tumorous and corresponding non-tumorous tissue

Intensities of immunoreactive bands normalized to β-actin (expression level of examined proteins) are presented in Table I. Irrespective of the VHL gene status, all tumorous tissues similarly expressed mTOR, eIF4E-BP1, and AMPK proteins (Figure 1, Figure 2, Figure 3). Only tumors with biallelic inactivation demonstrated lower mTOR and higher eIF4E-BP1 expression in comparison to control tissue (P = 0.002 for both).

Expression level of REDD1 protein is influenced by the VHL gene. Tumors with monoallelic inactivation of VHL underexpressed REDD1 in comparison to both wt tumors (P = 0.042) and tumors with biallelic gene inactivation (P < 0.005) as well as to control tissue (P = 0.004). On the contrary, REDD1 expression in tumorous tissue with biallelic inactivation of VHL was higher than in control tissue (P = 0.008) (Figure 4).

Wild-type ccRCCs expressed significantly higher amounts of PDK3 protein in comparison to tumors with biallelic inactivation of the VHL gene (P = 0.012) and control renal tissue (P = 0.016) (Figure 5).

Correlations and multivariate linear regression analysis
In tumors with wt VHL gene expression of PDK3 protein was in a positive correlation with AMPK protein (r = 0.987, P = 0.013). Multivariate linear regression analysis has shown that 97.4% of variability in the expression level of PDK3 can be explained by

| VHL        | mTOR     | eIF4E-BP1 | AMPK    | REDD1    | PDK3    |
|------------|----------|-----------|---------|----------|---------|
| WT         | 0.83 ± 0.45 | 0.85 ± 0.35 | 0.59 ± 0.30 | 0.37 ± 0.15 | 1.06 ± 0.3a,c |
| M/LOH      | 0.41 ± 0.19 | 0.47 ± 0.17 | 0.39 ± 0.13 | 0.06 ± 0.05a,b,c | 4.33 ± 3.79  |
| M+LOH      | 0.21 ± 0.06a | 0.80 ± 0.12a | 0.70 ± 0.18 | 0.45 ± 0.10a | 0.32 ± 0.08  |
| C          | 0.50 ± 0.07 | 0.55 ± 0.05 | 0.50 ± 0.08 | 0.25 ± 0.05 | 0.36 ± 0.06  |

aP < 0.05 vs. C; bP < 0.05 vs. WT; cP < 0.05 vs. M+LOH; WT, wild-type VHL; M/LOH, VHL-mutated or loss of heterozygosity of 3p locus; M+LOH, VHL-mutated and loss of heterozygosity of 3p locus; C-control renal tissue.
Figure 1 Expression level of mTOR in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for mTOR in tumorous and non-tumorous tissue. B) Tumors with biallelic inactivation underexpressed mTOR in comparison to control tissue. There was no significant difference regarding \( VHL \) mutational status. WT, wild-type \( VHL \); M/LOH, \( VHL \)-mutated or loss of heterozygosity of 3p locus; M+LOH, \( VHL \)-mutated and loss of heterozygosity of 3p locus; C, control renal tissue. (*\( P = 0.002 \) vs. control)

Figure 2 Expression level of eIF4EBP1 in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for eIF4EBP1 in tumorous and non-tumorous tissue. B) Tumors with biallelic inactivation underexpressed eIF4EBP1 in comparison to control tissue. Abundance of eIF4EBP1 was not influenced by \( VHL \) gene mutational status. WT, wild-type \( VHL \); M/LOH, \( VHL \)-mutated or loss of heterozygosity of 3p locus; M+LOH, \( VHL \)-mutated and loss of heterozygosity of 3p locus; C, control renal tissue. (*\( P = 0.002 \) vs. control)
Figure 3 Expression level of AMPK in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for AMPK in tumorous and non-tumorous tissue. B) Similar expression of AMPK irrespectively of VHL gene mutational status. WT, wild-type VHL; M/LOH, VHL-mutated or loss of heterozygosity of 3p locus; M+LOH, VHL-mutated and loss of heterozygosity of 3p locus; C, control renal tissue.

Figure 4 REDD1 expression level in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for REDD1 in tumorous and non-tumorous tissue. B) Tumors with monoallelic inactivation of VHL underexpressed REDD1 in comparison to wtVHL and tumors with biallelic inactivation of VHL, and to control renal tissue. REDD1 expression was higher in tumors with biallelic VHL inactivation than in control tissue. WT, wild-type VHL; M/LOH, VHL-mutated or loss of heterozygosity of 3p locus; M+LOH, VHL-mutated and loss of heterozygosity of 3p locus; C, control renal tissue. (*P < 0.001 vs. control, #P = 0.042 vs. wtVHL; ‡P < 0.005 vs. M+LOH)
variations in AMPK expression (Coefficient B ± SE, 0.778 ± 0.09, $P < 0.001$).

**Discussion**

Tumors with biallelic VHL gene inactivation exhibited higher expression levels of REDD1 than those with monoallelic VHL inactivation and corresponding control tissues. While mTOR expression was similar among tumor tissues, it was lower in tissues with biallelic inactivation than in controls. We also demonstrated that biallelic inactivation is accompanied with lower Fuhrman’s grade in comparison to wt ccRCCs. This is in line with the results of Kucejova et al. (16) who demonstrated that VHL gene inactivation is crucial for REDD1 overexpression. They also provide the evidence for the presence of a positive relation between Fuhrman’s grade and mTORC1 activity. Thus, underexpression of mTOR with upregulation of REDD1 suggests dominant signaling through mTORC2. This could be supported by the presence of HIF-2α in VHL negative tumorous tissues, whose synthesis is hinged on mTORC2 activity (26). Furthermore, these tumors have increased expression levels of elf4EBP1 in comparison to corresponding healthy tissues. It has been shown that both hypophosphorylation of elf4EBP1 and overexpression of elf4EBP1 as a cell-protective mechanism in stress conditions inhibit mTORC1 activity and restrain protein synthesis (27). The interplay between elf4EBP1 and mTORC1 during energy deprivation appears to be the consequence of REDD1 overexpression in VHL negative tumors, indicating that balancing between mTORC1 and mTORC2 could depend on pVHL (28).

Similar expression levels of REDD1 in wt VHL and tumors with biallelic inactivation of VHL may indicate functional inactivation of pVHL rendered by hypoxia (29–31). Higher Fuhrman’s grade in wt VHL tumors than in tumors with biallelic inactivation of VHL accompanied with similar expressions of REDD1 and mTOR may reflect resistance of mTORC1 to REDD1 inhibition (16, 32).

Along with incapability to increase REDD1 expression, VHL haploinsufficient tumors exhibited unexpectedly decreased levels of the aforementioned REDD1. In spite of the proposed regulation of HIFs by negative feedback control (33), our results suggest that decreased levels of REDD1 in tumors with monoallelic inactivation of VHL are not only transcriptionally regulated by the HIFs whose expression is VHL independent (34). Possible explanations for the depletion of REDD1 could be the presence of Redd1 mutations, which are apparently rare (16). Additionally, it was reported that miR-221 which binds to 3’-UTR of Redd1 transcript is overexpressed in patients with ccRCCs (35–38). Besides, tumorous expression

![Figure 5](image-url)
of REDD1 and TSC1 (Hamartin) follows the same pattern of expression and VHL dependency (25). Of note is that REDD1, TSC1 and TSC2 (Tuberin) degradation is regulated by the mutual ubiquitin-dependent proteasomal system comprised of CUL4A-DDB1-ROC1-b-TRCP E3 ligase complex mediated by phosphorylation activity of GSK3β (Glycogen Synthase Kinase 3β) (39, 40). Potential link between the expression of REDD1 and TSC1 in ccRCC could be GSK3β since it is also involved in pVHL phosphorylation that affects its HIF-independent functions (32, 41, 42).

Results of our study also implicate that the metabolic profile of ccRCCs depends on the VHL mutational status. Expression of PDK3 in wtVHL tumors was higher in comparison to tumors with biallelic inactivation of VHL and corresponding control tissue. Overexpression of PDK3 in wt VHL tumors suggests glycolysis as a dominant metabolic pathway for ATP production. This finding highlights the importance of determination of VHL mutational status for the most efficient therapeutic strategy (43). Almost all (97.4%) variability in PDK3 expression is explained by the amount of AMPK in these tumors reflecting a positive functional relation between the two molecules in response to hypoxia (44).

Although small sample size was a limitation of the present study, significant differences in the expression profile of wtVHL and tumors with biallelic inactivation of VHL may provide advantages in the therapy of ccRCC and should be considered for further investigation.

In conclusion, expressions of mTOR, elf4EBP1 and AMPK proteins were VHL independent in ccRCC. Our study provides additional evidence that VHL mutational status affects REDD1 expression in these tumors. For the first time, we have shown that PDK3 has VHL dependent expression in sporadic ccRCC.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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