

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

VHL ZAVISNA EKSPRESIJA PROTEINA REDD1 I PDK3  
U SVETLOĆELIJSKOM KARCINOMU BUBREGA

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### 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, eIF4E-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* (wt*VHL*) gene obtained from patients who underwent total nephrectomy. The expressions of target proteins were assessed using Western blot.

**Results:** Expressions of mTOR, eIF4EBP1 and AMPK were *VHL* independent. Tumors with monoallelic inactivation of *VHL* underexpressed REDD1 in comparison to wt*VHL* 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 wt*VHL* ccRCC, multivariate linear regression analysis revealed that 97.4% of variability in PDK3 expression can be explained by variations in AMPK amount.

### Kratak sadržaj

**Uvod:** Sporadični svetloćelijski karcinom bubrega asociran je sa mutacijama u genu *VHL*, povišenom aktivnošću mTOR signalnog puta i glikolitičkim metabolizmom. Cilj ove studije bio je da se ispita efekat mutacionog statusa gena *VHL* na nivo ekspresije proteina mTOR, eIF4E-BP1, AMPK, REDD1 i PDK3.

**Metode:** Ukupni proteini izolovani su iz uzorka tumorskog tkiva sa bialelnom inaktivacijom gena *VHL* ( $n = 21$ ), uzoraka sa monoalelnom inaktivacijom ( $n = 10$ ) i tumora sa neizmjenjenim genom *VHL* (wt*VHL*) ( $n = 6$ ) dobijenih od bolesnika sa karcinomom bubrega nakon totalne nefrektomije. Nivo ekspresije ispitivanih proteina utvrđen je Western blot metodom.

**Rezultati:** Nisu detektovane razlike u nivou ekspresije proteina mTOR, eIF4EBP1 i AMPK u zavisnosti od mutacionog statusa gena *VHL*. Tumori sa monoalelnom inaktivacijom gena *VHL* imali su povišen nivo ekspresije proteina REDD1 u poređenju sa wt*VHL* tumorima ( $P = 0,042$ ), tumorima sa bialelnom inaktivacijom gena *VHL* ( $P < 0,005$ ) i kontrolnim tkivom ( $P = 0,004$ ). Dodatno, ekspresioni nivo REDD1 proteina je bio viši u tumorima sa bialelnom inaktivacijom gena *VHL* u odnosu na kontrolno tkivo ( $P = 0,008$ ). Nivo ekspresije proteina PDK3 u wt*VHL* tumorima je bio povišen u odnosu na tumore sa bialelnom inaktivacijom gena *VHL* ( $P = 0,012$ ) i kontrolno tkivo bubrega ( $P = 0,016$ ). U tumorima sa wt*VHL* genom, multivarijantnom linearnom regresio-

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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, eIF4EBP1 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 eIF4E-BP1 (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 isoform in different carcinomas, but there is no information about the expression level of PDK3 isoform in ccRCCs (21–23).

In the present study, we evaluated the effect of *VHL* mutational status on the expression level of mTOR, eIF4E-BP1, 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 in-

formed 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.

**Zaključak:** Ekspresioni nivo proteina mTOR, eIF4EBP1 i AMPK nezavisan je od mutacionog statusa gena *VHL*. Ovom studijom je prvi put pokazano da nivo ekspresije proteina PDK3 zavisi od mutacionog statusa gena *VHL* i pruženi su dodatni dokazi da mutacioni status gena *VHL* utiče na nivo ekspresije proteina REDD1 u svetloćelijskom karcinomu bubrega.

**Ključne reči:** svetloćelijski karcinom bubrega, PDK3, mTOR, REDD1, gen *VHL*

### *Protein isolation and Western blot*

Proteins were extracted from tumorous 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-Tris 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-eIF4EBP1 (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-

ature and washed in PBST. Proteins were visualized by chemiluminescence (Lumi-light<sup>PLUS</sup> Western Blotting kit, Roche Applied Science, Mannheim, Germany). After visualization, membranes were stripped with 0.2 mol/L NaOH, and re probed with another primary antibody or anti- $\beta$ -actin antibody. The intensities of immunoreactive bands were determined using ImageQuant 5.2 software (GE Healthcare, Little Chalfont, UK). Protein loading was normalized to  $\beta$ -actin (24).

#### Statistical analysis

All data are presented as mean  $\pm$  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<sup>®</sup> 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.

Tumors with wt *VHL* gene have shown significantly higher Fuhrman's nuclear grade ( $2.8 \pm 0.2$ ) in comparison to tumors with monoallelic ( $1.8 \pm 0.2$ ;  $P = 0.026$ ) and biallelic ( $2.1 \pm 0.2$ ;  $P = 0.04$ ) inactivation of the *VHL* gene.

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

Intensities of immunoreactive bands normalized to  $\beta$ -actin (expression level of examined proteins) are presented in Table 1. Irrespectively of the *VHL* gene status, all tumorous tissues similarly expressed mTOR, eIF4EBP1, and AMPK proteins (Figure 1, Figure 2, Figure 3). Only tumors with biallelic inactivation demonstrated lower mTOR and higher eIF4EBP1 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).

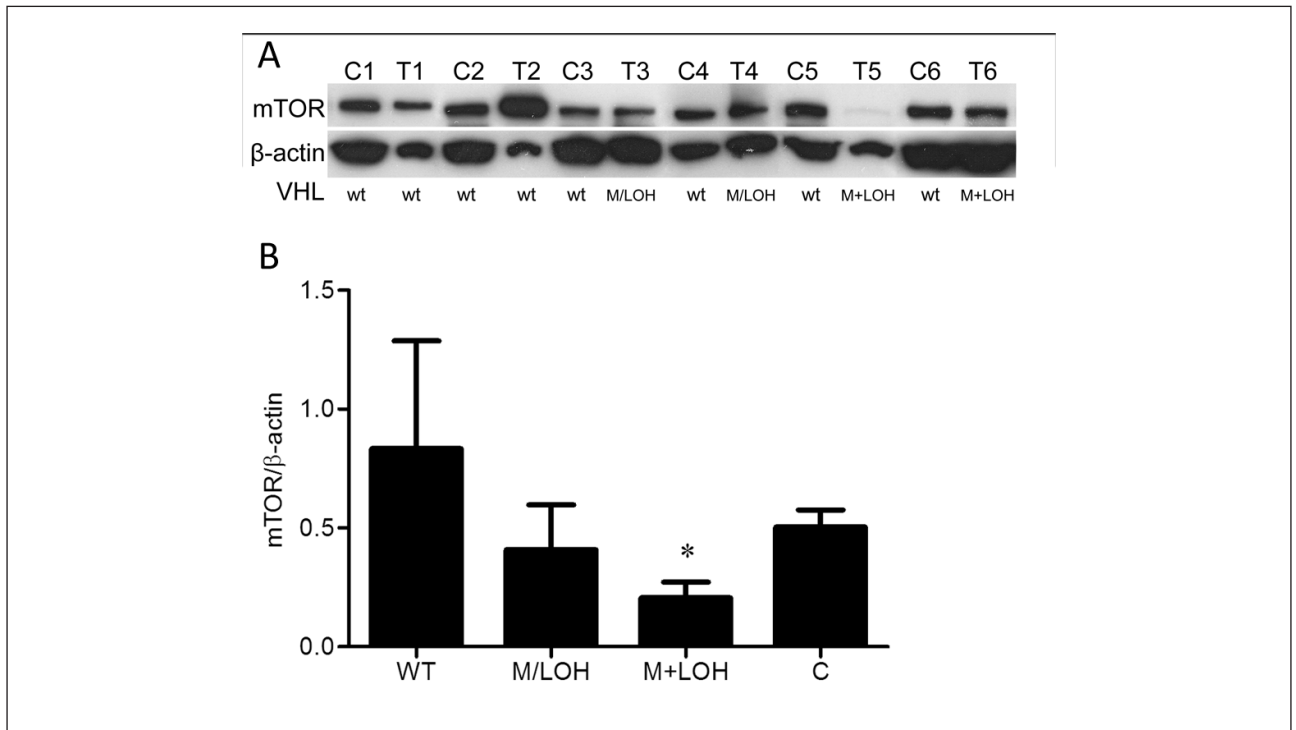
#### Correlations and multivariate linear regression analysis

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

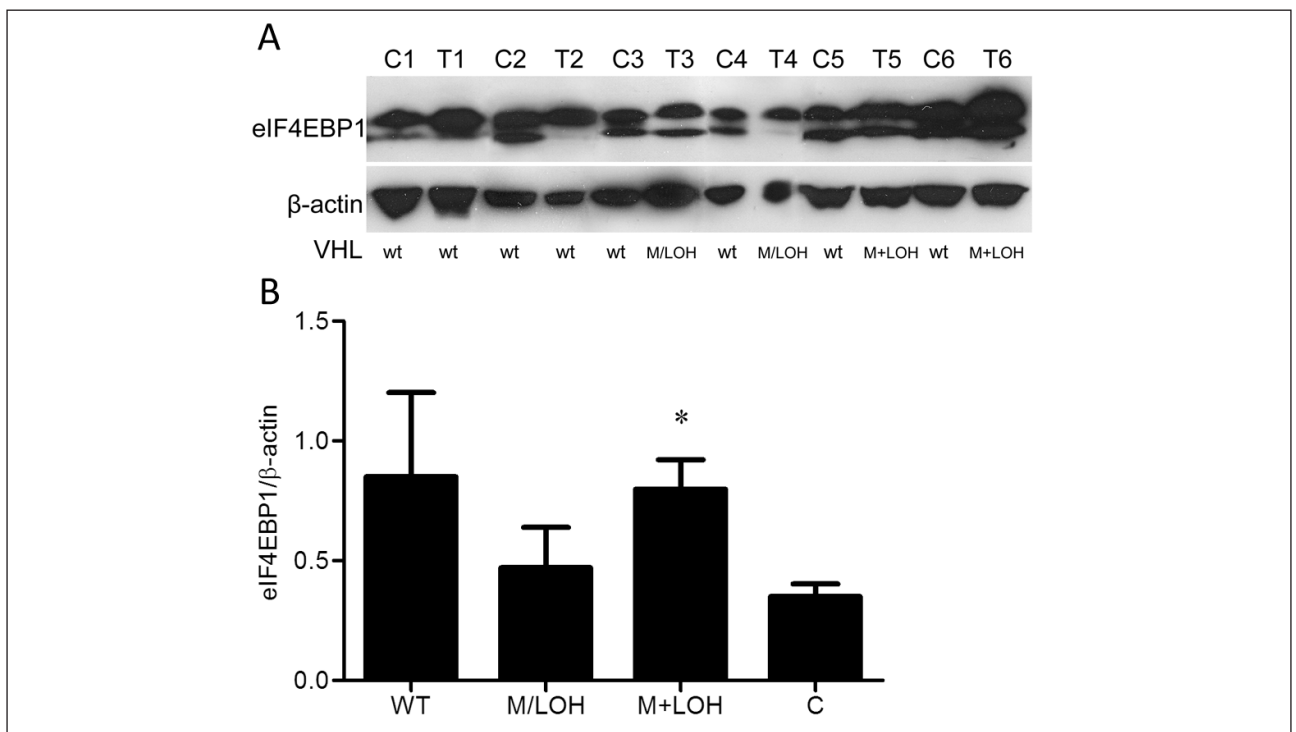
**Table 1** Expression level of mTOR, eIF4E-BP1, AMPK, REDD1 and PDK3 proteins in ccRCCs and control renal tissue.

<i>VHL</i>	mTOR	eIF4E-BP1	AMPK	REDD1	PDK3
WT	$0.83 \pm 0.45$	$0.85 \pm 0.35$	$0.59 \pm 0.30$	$0.37 \pm 0.15$	$1.06 \pm 0.3_{a,c}$
M/LOH	$0.41 \pm 0.19$	$0.47 \pm 0.17$	$0.39 \pm 0.13$	$0.06 \pm 0.03_{a,b,c}$	$4.33 \pm 3.79$
M+LOH	$0.21 \pm 0.06_a$	$0.80 \pm 0.12_a$	$0.70 \pm 0.18$	$0.45 \pm 0.10_a$	$0.32 \pm 0.08$
C	$0.50 \pm 0.07$	$0.35 \pm 0.05$	$0.50 \pm 0.08$	$0.25 \pm 0.05$	$0.36 \pm 0.06$

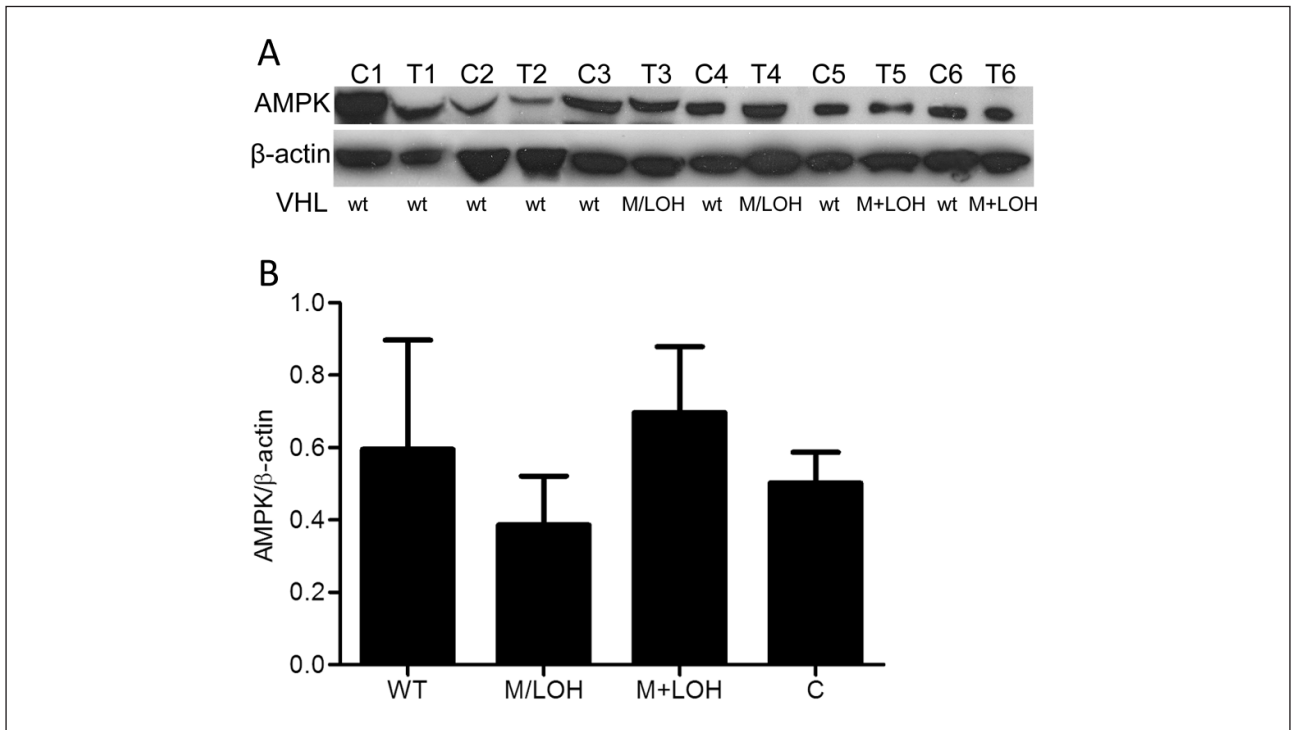
<sup>a</sup> $P < 0.05$  vs. C; <sup>b</sup> $P < 0.05$  vs. WT; <sup>c</sup> $P < 0.05$  vs. M+LOH; WT, wild-type *VHL*; M/LOH, *VHL*-mutated or loss of heterozygosity of 3p locus; M+LOH, *VHL*-mutated and loss of heterozygosity of 3p locus; C-control renal tissue.



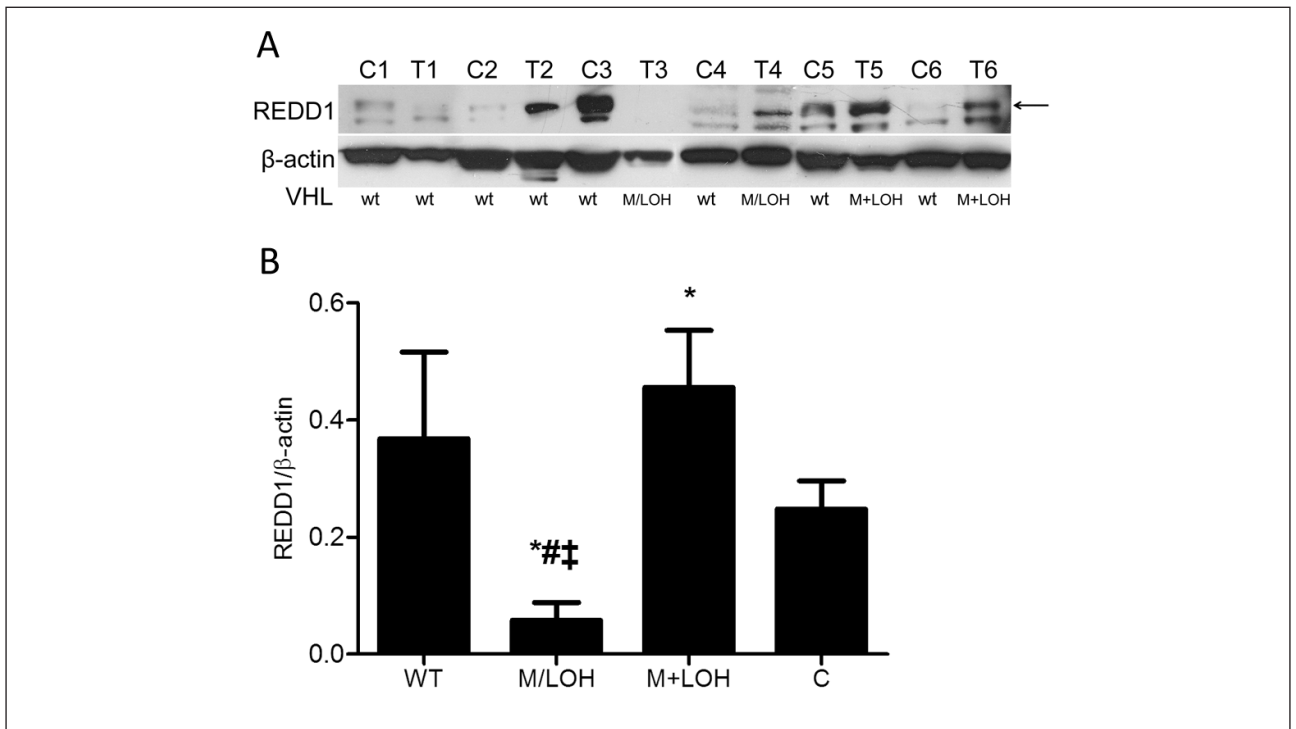
**Figure 1** Expression level of mTOR in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for mTOR in tumorous and non-tumorous tissue. B) Tumors with biallelic inactivation underexpressed mTOR in comparison to control tissue. There was no significant difference regarding *VHL* mutational 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)



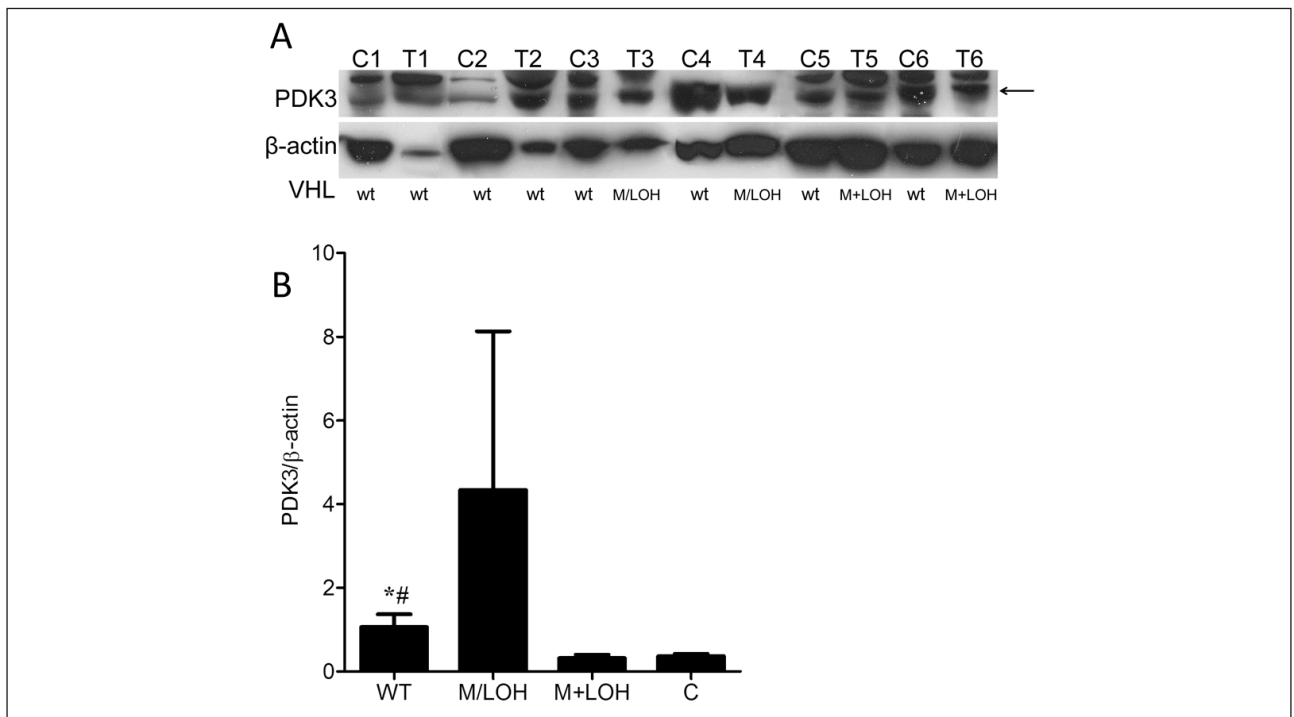
**Figure 2** Expression level of eIF4EBP1 in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for eIF4EBP1 in tumorous and non-tumorous tissue. B) Tumors with biallelic inactivation underexpressed eIF4EBP1 in comparison to control tissue. Abundance of eIF4EBP1 was not influenced 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 wt*VHL* 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. wt*VHL*; ‡ $P < 0.005$  vs. M+LOH)



**Figure 5** Expression level of PDK3 in ccRCCs and corresponding non-tumorous tissue samples. A) Western blot for PDK3 in tumorous and non-tumorous tissue. B) wt *VHL* tumors expressed higher amounts of PDK3 in comparison to tumors with biallelic *VHL* inactivation and to 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. (\* $P = 0.016$  vs. control; # $P = 0.012$  vs. M+LOH)

variations in AMPK expression (Coefficient B  $\pm$  SE,  $0.778 \pm 0.09$ ,  $P < 0.001$ ).

## 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-2 $\alpha$  in *VHL* negative tumorous tissues, whose synthesis is hinged on mTORC2 activity (26). Furthermore, these tumors have increased expression levels of eIF4EBP1 in comparison to corresponding healthy tissues. It has been shown that both hypophosphorylation of eIF4EBP1 and overexpression of eIF4EBP1 as a cell-protective mechanism in stress conditions

inhibit mTORC1 activity and restrain protein synthesis (27). The interplay between eIF4EBP1 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



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 GSK3 $\beta$  (Glycogen Synthase Kinase 3 $\beta$ ) (39, 40). Potential link between the expression of REDD1 and TSC1 in ccRCC could be GSK3 $\beta$  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 wt*VHL* 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 pos-

itive 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 wt*VHL* 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, eIF4EBP1 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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