VHL loss predicts response to Aurora kinase A inhibitor in renal cell carcinoma cells

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Abstract. The majority of molecular targets of anticancer agents are limited to a subset of patients, and therefore identification of more specific biomarkers that can be used to improve clinical outcomes is of increasing interest. The present study showed that von Hippel-Lindau tumor suppressor (VHL) tumor-suppressor activity may influence the therapeutic response to Aurora kinase A (AURKA) inhibitors in human renal cell carcinoma (RCC). VHL protein (pVHL) expression was evaluated by immunoblotting in the human RCC cell lines CAKI, ACHN, 786-O, 769-P and A498. The anti-tumor activities of alisertib, an AURKA-specific chemical inhibitor, were detected by Cell Counting Kit-8 assay *in vitro* and mouse xenograft model *in vivo*. Additionally, the VHL-mediated anti-tumor activity was assessed in 769-P and CAKI cells via the loss or gain of VHL. The results revealed that VHL-deficient 786-O, 769-P and A498 cells were sensitive to alisertib. By contrast, alisertib-resistant CAKI and ACHN cells expressed the wild type VHL gene. In addition, rescue or knockdown of VHL was observed to increase or decrease alisertib anti-proliferation activity, respectively, in RCC cells. The inverse correlation between the VHL gene expression profile and alisertib sensitivity was further confirmed in human cancer xenografts models. Taken together, these results suggested that VHL loss could potentially serve as a biomarker for predicting the efficacy of AURKA inhibitors.

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Introduction

Around 208,500 new cases of kidney cancer are diagnosed annually worldwide (1). It has been predicted that around 63,340 new cases of kidney cancer will occur, and about 14,970 patients will die from kidney cancer in the United States in 2018 (NIH). In China, the kidney cancer incidence has been rising steadily in recent years (MOHC, Ministry of Health of The People's Republic of China). Renal cell carcinoma (RCC), the most common type of kidney cancer in adults, is responsible for approximately 90-95% of cases (2,3). As 25-30% of RCC patients exhibit metastatic spread by the time they are diagnosed, drug treatments targeting metastasis are needed during early disease management. In this regard, Aurora kinase A (AURKA) inhibitors have been shown to inhibit the growth and spread of RCC (4-6).

AURKA, a serine-threonine-specific protein kinase, is a member of the Aurora kinase (AURK) family, the members of which serve as key regulators of mitosis; essential for accurate and equal segregation of genomic material from parent to daughter cells (7). Humans have three classes of Aurora kinases, namely AURKA, AURKB and AURKC. AURKA is a 403 amino-acid protein with a calculated molecular weight of 48 kDa (8). This protein has an N-terminal regulatory domain and C-terminal catalytic domain. Two amino acid sequences, the A-box in the regulatory domain and the destruction-box (D-box) in the catalytic domain, are necessary for recognition of AURKA by the APC/C complex, which mediates its degradation at the end of mitosis or in G1 (9). Phosphorylation of a threonine residue within the activation loop in the catalytic domain is critical for Aurora kinase activity (10-12). AURKA is mainly localized at spindle poles and the mitotic spindle during mitosis, where it regulates centrosomes, spindles and kinetochores. Recent studies have revealed that AURKA is frequently overexpressed in several cancer cells, indicating its involvement in tumor initiation and development (5,13-15). Certain ongoing clinical trials and primary studies are assessing the unique therapeutic potential of AURKA-targeted therapy for RCC. However, RCC is relatively resistant to chemotherapy. Therefore, identifying early-stage predictive biomarkers in RCC patients is urgently needed.

In the present study, we showed that RCC cells with loss of the VHL gene were more sensitive to AURKA inhibitor.

Moreover, we found that elevated pVHL levels improved AURKA degradation, as a novel mechanism for the role of pVHL in regulating AURKA.

Materials and methods

Cell culture and reagents. CAKI, ACHN, 786-O, 769-P and A498 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in appropriate medium as suggested by the ATCC. Cells were incubated in a humidified atmosphere of 95% air plus 5% CO_2 at 37˚C. Alisertib and MG-132 were obtained from Selleck Chemicals, (Houston, TX, USA). Cycloheximide (CHX) was purchased from Solarbio Science and Technology (Beijing, China).

Immunoblotting. After different types of treatment were performed, cells were harvested and resuspended in RIPA lysis buffer (weak). Equivalent amounts of proteins were analyzed by SDS-PAGE. Appropriate antibodies against VHL (1:1,000), AURKA (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), GAPDH (1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used. Proteins were visualized with peroxidase-coupled secondary antibody from Sigma-Aldrich; Merck KGaA, (Darmstadt, Germany), using ECL solution for detection.

Animals and anti-tumor activity assay in vivo. BALB/Ca-nu/nu mice aged 4-5 weeks were obtained from Zhejiang Academy of Medical Sciences. The animals were housed in sterile cages under laminar airflow hoods in a specific pathogen-free room with a 12-h light and 12-h dark schedule, and fed autoclaved chow and water ad libitum. Animal experiments were performed according to the institutional ethical guidelines of animal care and were approved by Taizhou University (no. TZYXY2016-302). The largest diameter of tumors developed in all mice examined is 1.745 cm. shRNA-transfected cells were transplanted subcutaneously into the flanks of nude mice. When the tumor volume reached about 100 mm^3, the mice were randomly assigned into control and treatment groups, 8 mice each group. Control groups were given vehicle, and treatment groups received alisertib at the indicated doses per os for five days per week for 3 weeks. Tumor volumes were measured twice per week. After treatment, animals were killed by cervical dissociation, and solid tumors were removed and weighed. The inhibition rate was calculated as [(average tumor weight of vehicle control group/average tumor weight of test group)/average tumor weight of normal saline group] X100%.

Knockdown of VHL by shRNA. The lentivirus vector pLKO.1 was used. The inserted shRNA-targeting *vhl* sequence was as follows: 5'-CCGGTATCACACTGCCAGTGTATACCTCGAG GTATACACTGGCAGTGTGATATTTTTG-3'. Lentiviral packaging was conducted in 293T cells following standard procedures. The shRNA was transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the instructions of the manufacturer.

Statistical analysis. The data were analyzed using SPSS v13.0 (SPSS, Inc., Chicago, IL, USA). The results were compared using one-way analysis of variance followed by Dunnett's post hoc test for multiple comparisons. All results are expressed as the mean ± standard deviation from three replicates. $P<0.05$ was considered to indicate a statistically significant difference.

Results

VHL gene expression profile and anti-proliferation effect of AURKA inhibitor in RCC cells. Immunoblot detection revealed that two RCC cell lines (ACHN and CAKI) expressed the wild type VHL gene, while the other three (A498, 769-P and 786-O) were VHL-deficient cells (Fig. 1A), consistent with reports by the National Cancer Institute using cDNA micro-arrays (genome-www.edu/nci60/).

Inhibition of cellular proliferation was evaluated using a CCK-8 kit (Beyotime Institute of Biotechnology, Haimen, China). In the present study, the AURKA-specific chemical inhibitor alisertib more readily inhibited the growth of ACHN and CAKI cells when compared with its effect in the other three RCC cell lines (Fig. 1B). IC50 values for alisertib in each cell line were as follows: ACHN, ~8 μmol/l; CAKI, ~7.5 μmol/l; 786-O, ~0.20 μmol/l; 769-P, ~0.25 μmol/l; A-498, ~0.33 μmol/l. We found a clear positive relationship between VHL gene expression and alisertib sensitivity (IC50) in the RCC cells. In other words, RCC cells expressing wild type VHL were more sensitive to alisertib (Fig. 1A and B). This Pearson's correlation was further confirmed in a xenograft animal model. As shown in Fig. 1C, alisertib displayed differential anti-tumor activity in CAKI and 769-P xenografts, with inhibitory rates of 36.0 and 60.7%, respectively.

Alteration in alisertib anti-proliferation activity by VHL. To confirm the positive relationship between the VHL gene expression profile and alisertib sensitivity in RCC cell lines, 769-P cells were stably transfected with pCDH-VHL plasmid; cells transfected with empty pCDH-vector served as the control. As shown in Fig. 2A, VHL protein could be detected in 769-P cells transfected with pCDH-VHL. Furthermore, 769-P cells transfected with pCDH-VHL were shown to be more resistant to alisertib than those transfected with empty vector (Fig. 2B), with the IC50 increasing from 0.25 to 1.25 μmol/l.

To further explore whether there is a causal relationship between VHL expression and alisertib sensitivity, CAKI cells were transfected with shRNA directed against VHL; cells transfected with empty vector as the control. Cells were harvested 72 h after transfection and extracts were prepared and analyzed by western blotting. Expression of the VHL gene decreased significantly after shRNA transfection (Fig. 3A). At 24 h after transfection with VHL shRNA or control vector, CAKI cells were treated with alisertib for 72 h. The cytotoxicity of alisertib was significantly higher for cells in the presence of VHL shRNA than for the control group (Fig. 3B), which indicated that decreasing the expression of VHL altered the anti-proliferation activity of alisertib.

This association was further confirmed in the animal model. As shown in Fig. 3C, alisertib displayed differential anti-tumor activity in the xenograft model of CAKI cells transfected with VHL-shRNA or control vector, with inhibitory rates of ~54 and ~32%, respectively (Fig. 3C).
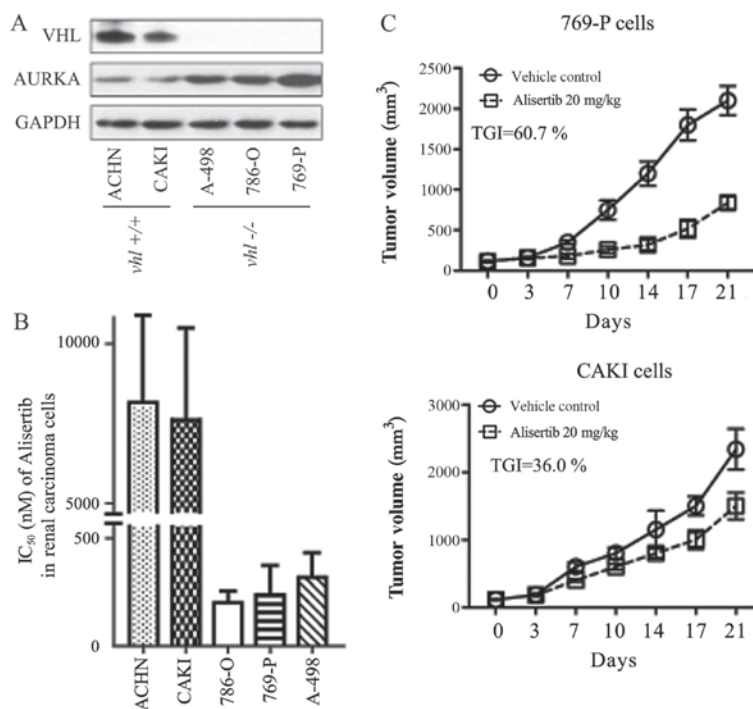
Figure 1. VHL expression profiles and alisertib anti-proliferative activities in RCC cells. (A) VHL and AURKA protein expression was detected by immunoblotting. GAPDH was employed as a loading control. (B) Anti-proliferative activity was assessed by Cell Counting Kit-8 assay following exposure to alisertib for 72 h. (C) Alisertib anti-tumor activity *in vivo*. VHL, von Hippel-Lindau tumor suppressor; RCC, renal cell carcinoma; AURKA, Aurora kinase A; IC50, half-maximal inhibitory concentration; TGI, tumor growth inhibition rate.
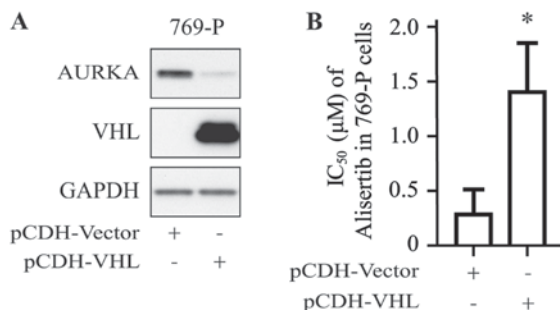

Figure 2. 769-P cells with VHL re-expression show resistance to alisertib. (A) VHL and AURKA protein expression in 769-P cells transfected with control or VHL plasmid was analyzed by immunoblotting. GAPDH was employed as a loading control. (B) The anti-proliferative activity of alisertib against cells was assessed by Cell Counting Kit -8 assay. *P<0.01 vs. vector. VHL, von Hippel-Lindau tumor suppressor; AURKA, Aurora kinase A; IC50, half-maximal inhibitory concentration.

VHL down-regulates AURKA in RCC cells. According to our results, we may conclude that VHL loss makes human RCC cells more sensitive to AURKA inhibitor. This increased sensitivity might be caused by VHL down-regulating AURKA via the HIF pathway. Consistent with previous reports (16,17), we showed that VHL down-regulated AURKA expression (Figs. 1A, 2A and 3A).

As reported by Xu *et al* (16), VHL inactivation induced AURKA expression in clear cell RCC (CCRCC) cells via the HIF pathway. In the present study, we also observed that hypoxic conditions could induce AURKA protein expression in RCC (Fig. 4A).

We have shown in a previous study that pVHL regulate AURKA directly via an HIF-independent pathway (17). As

shown in Fig. 4B and C, in the present study we confirmed that VHL can promote AURKA degradation. Moreover, the proteasome inhibitor, MG-132, blocked pVHL-induced AURKA degradation. Interestingly, the AURKA protein had a shorter half-life in 769-P cells re-expressed with VHL compared with that in control cells.

Discussion

In this study, we showed that 786-O, 769-P and A498 cells deficient in the VHL gene were sensitive to alisertib, an AURKA-specific chemical inhibitor. Conversely, alisertib-resistant CAKI and ACHN cells expressed the wild type VHL gene. Re-expression or knockdown of VHL reversed the anti-proliferation activity of alisertib in RCC cells. The inverse association between VHL gene expression profile and alisertib sensitivity was confirmed in human cancer xenografts models. Taken together, we suggest that VHL loss could potentially serve as a biomarker for predicting the efficacy of AURKA inhibitors.

It is well known that VHL protein serves as a substrate recognition component of an E3-ubiquitin ligase complex that targets hypoxia inducible factor (HIF) for ubiquitination and degradation (18). In addition to HIF-α, pVHL interacts with certain other proteins and has multiple functions, including in microtubule dynamics, cell proliferation and growth, neuronal apoptosis, extracellular matrix deposition, DNA damage response/repair and cilia maintenance. It has been extensively reported in human cancers that loss of VHL is a predictive biomarker for the efficacy of neoadjuvant therapy (19,20). In the present study, we proposed the potential of VHL to be a predictive marker of response to AURKA inhibitors and tested
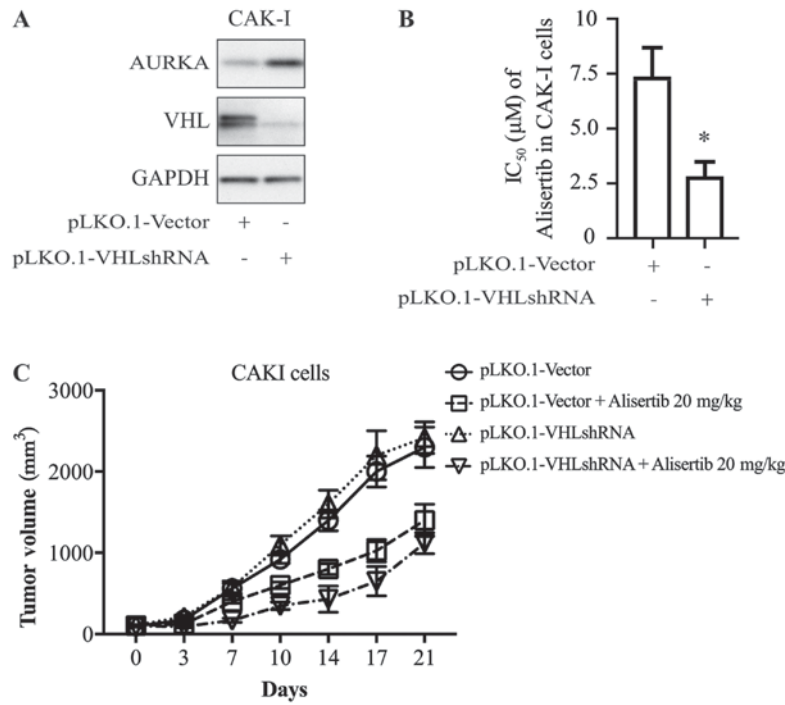
Figure 3. shRNA knockdown of VHL in CAKI cells confers decreased alisertib sensitivity. (A) VHL and AURKA protein expression in CAKI cells transfected with control or VHL shRNA was analyzed by immunoblotting. GAPDH was employed as a loading control. (B) The anti-proliferative activity of alisertib against cells was assessed by Cell Counting Kit -8 assay. *P<0.01 vs. vector. (C) Inhibition of tumor growth by alisertib in xenografts of CAKI cells transfected with control or VHL shRNA. VHL, von Hippel-Lindau tumor suppressor; AURKA, Aurora kinase A; IC50, half-maximal inhibitory concentration; shRNA, short hairpin RNA.
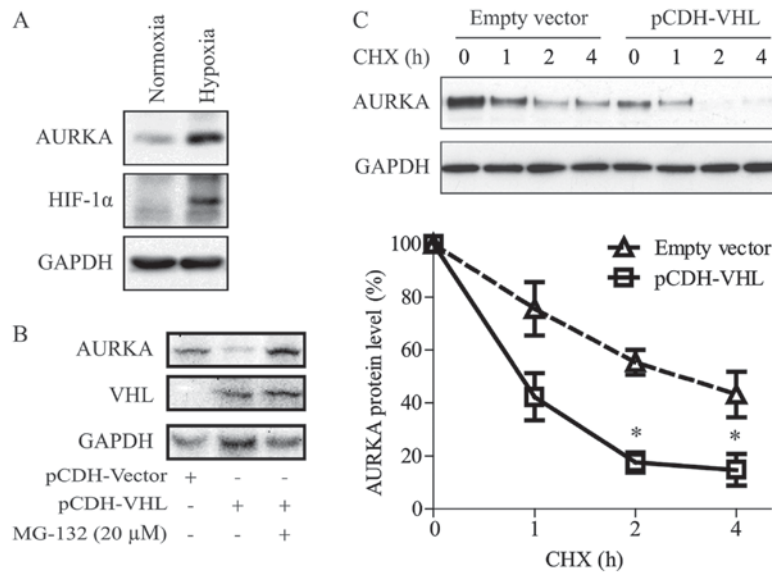

Figure 4. VHL regulates AURKA levels via HIF-dependent and -independent pathways. (A) Hypoxia could induce AURKA protein upregulation upon analysis of HIF-1 and AURKA protein expression in CAK-I cells by immunoblotting; GAPDH was employed as a loading control. (B) The 26S proteasome inhibitor MG132 (10 μM) rescued the downregulation of AURKA in 769-P cells re-expressed with pVHL. *P<0.01 vs. empty vector. (C) pVHL promoted AURKA degradation. 769-p cells were transfected with either pVHL or vector plasmid. Cells were treated with 100 μg/ml CHX and harvested at the indicated time points, and cell lysates were prepared. Proteins from cell lysates were subjected to western blotting with anti-AURKA and anti-GAPDH antibodies. Relative protein levels were plotted from the integrated optical density of the AURKA bands on the western blot (lower panel). VHL, von Hippel-Lindau tumor suppressor; AURKA, Aurora kinase A; IC50, half-maximal inhibitory concentration; HIF, hypoxia inducing factor; pVHL, VHL protein; CHX, cycloheximide.

our hypothesis *in vitro* and *in vivo*. We further speculated that pVHL regulated AURKA levels via HIF-dependent and HIF-independent pathways. As it is technically feasible and reproducible to measure VHL loss in patients, which should be easier than detecting AURKA expression levels, VHL meets the criteria to serve as a predictive marker of efficacy of alisertib-based therapy.

Our data supported that VHL loss could be potentially applied as a biomarker to predict the efficacy of AURKA inhibitors. Additional pre-clinical and clinical trials are needed

to validate our findings. It is necessary to confirm our primary data with results obtained from the patients' samples (e.g. PDTX model) and correlate the results with the clinic pathological data.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XFD, GC and YLW designed the research. XFD, JZ and GC performed the research. XFD, GC and YLW analyzed the data, and GC and YLW wrote the manuscript.

Ethics approval and consent to participate

Animal experiments were performed according to the institutional ethical guidelines of animal care and were approved by Taizhou University (no. TZYXY2016-302).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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