DAB2IP stabilizes p27Kip1 via suppressing PI3K/AKT signaling in clear-cell renal cell carcinoma

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Abstract

Renal cell carcinoma (RCC) is the most lethal of the urologic malignancies. We previously discovered that DAB2IP, a novel Ras-GTPase-activating protein, was frequently epigenetically silenced in RCC, and DAB2IP loss was correlated with the overall survival of RCC patients. In this study, we determined the biological functions of DAB2IP in RCC and its potential mechanisms of action. Correlations between DAB2IP expression level and RCC tumor size and patient survival were analyzed, and the results showed that RCC patients with high DAB2IP mRNA level exhibited smaller tumor size and better survival than the patients with low DAB2IP. Compared to control cells, DAB2IP knockdown (KD) cells showed enhanced cell proliferation, promoted G1/S phase cell cycle progression and decreased expression of p27. Mechanism studies demonstrated that loss of DAB2IP promoted p27 protein phosphorylation, cytosolic sequestration and subsequently ubiquitination-mediated degradation in RCC cells. Further studies confirmed that the proline-rich domain in C terminal (CPR) of DAB2IP suppressed AKT phosphorylation and induced p27 phosphorylation on S10. Hence, DAB2IP is essential for p27 protein stabilization in RCC, which is at less partly mediated by PI3K/AKT signaling pathway.

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer, more than 200,000 new cases and 100,000 deaths are estimated to occur worldwide[1]. This diease is characterized by substantial genetic heterogeneity, distinctive histological feature and clinical phenotype. Clear cell RCC (ccRCC) is the major subtype of RCC which accounts for approximately 75% of the cases. Systemic genome screenings of ccRCC have identified frequent alterations in ccRCC, such as, VHL, BAP1, PBRM1, SETD2 and PIK3CA, which are associated with ubiquitin-mediated proteolysis, chromatin-remodeling, histone methylation and protein kinase activation[2]. These alterations in proto-oncogene and anti-oncogene leads to dysregulated of signal transduction that underlies abnormal cell growth and metabolism.

We previously employed a yeast two-hybrid system to identify DAB2IP, a Ras GTPase-activating protein (GAP) that interacts with the N-terminal domain of DOC-2/DAB2 and has distinct cellular functions such as modulating different signal cascades associated with cell proliferation, survival, apoptosis and metastasis[3]. Genetic variants of DAB2IP are associated with increased risk of prostate and lung cancer[4, 5]. Recent studies have demonstrated that DAB2IP is down-regulated in multiple types of cancer caused by epigenetic modification (such as DNA methylation or histone deacetylation) and others[6]. It appears that DAB2IP functions as a tumor suppressor, since loss of this gene promotes tumor development and progression. Profile of DAB2IP in human fetal tissues indicated that DAB2IP is positively expressed during fetal development of kidney, especially in kidney tubule epithelial cells[7]. We recently report that DAB2IP is highly expressed in adult normal kidney tubule cells but lost in about 50% of ccRCC tissues[8]. In the present study, we further explored the biological functions of DAB2IP in ccRCC, and found that loss of DAB2IP was associated with increased RCC growth and poor patient survival. Mechanistically, loss of DAB2IP could activate AKT, thus promoting the phosphorylation and degradation of a cell-cycle inhibitor p27 ^{Kip1}.

Materials And Methods

Human specimens and Immunohistochemistry (IHC)

With approval of the institutional review board, 90 ccRCC patients with treated with nephrectomy were included in the study. All pathological specimens were processed according to standard pathological procedures. Tissue microarrays (TMA) were constructed from paraffin embedded blocks for immunohistochemical study. For DAB2IP, p27 and Ki-67 staining, sections were firstly deparaffinized, rehydrated and subjected to heat-induced antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were then incubated with appropriate primary antibodies, horseradish-peroxidase–labeled dextran polymer (DAKO EnVision^{TM}, Carpinteria, CA) and developed with 3,3'-diaminobenzidine chromogen followed by counter staining with hematoxylin.

Cell cultures

HK-2 and 786-0 cells were maintained in RPMI1640 medium (Gibco, Santa Clara, CA) supplied with 10% fetal bovine serum (FBS), ACHN and A498 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplied with 10% FBS. Stable cell sublines (i.e., Con and KD) were maintained in appropriate original medium supplemented with 0.3 μg/ml puromycin. All the cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

Cell viability assay

Cells were re-suspended in 100 μl of appropriate medium with 0.5% FBS and plated in 96-well plates at a concentration of 1500 cells/well. Cells were cultured for 48 hour (hr), and 20 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well for another 4 hr incubation. Thereafter, the medium containing MTT was removed, and 150 μl DMSO was added to solubilize the formazan crystals. The absorbance (OD) of each well was then measured at a wavelength of 590 nm by a microplate autoreader (Bio-Tek Instruments, Vinooski, VT).

Cell cycle assay

Cells were fixed in cold ethanol overnight and then washed with phosphate buffered saline with 0.5% Tween 20 (PBST) for 3 times. Cells were stained with 50 μg/mL propidium iodide for 1 hr and immediately analyzed by a fluorescence-activated cell-sorting scanning (FACS) flow cytometer (BD Biosciences, NJ).

Plasmid constructs and transfection

Various expression plasmids for DAB2IP and its mutants were described previously [9]. Wildtype (WT) p27 cDNA cloned into pcDNA3.1/myc-His expression vector was obtained from Dr. Claret (The University of Texas M.D. Anderson Cancer Center) [10]. A phosphomimetic mutation, S10A, was generated in the WT p27 cDNA plasmid by oligonucleotide-directed mutagenesis and polymerase chain reaction using a

QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Primers used for the mutagenesis were: F- 5'GCGAGTGTCTAACGGGGCCCCTAGCCTGGAGCG3' and R- 5' CGCTCCAGGCTAGGGGCCCCGTTAGACACTCGC3'. The mutation was confirmed by sequencing. Transfections were performed using Lipofectamine LTX and PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real-time RT-PCR

Cell total RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA) and 1 μg RNA was reverse-transcribed using iScript™ cDNA Synthesis kit from Bio-Rad (Hercules, CA) according to the manufacturer's instructions. Real-time PCR analysis was set up with SYBR Green qPCR Supermix kit (Invitrogen, Carlsbad, CA) and carried out in the iCycler thermal cycler. The relative level of mRNA expression of each gene was determined by normalizing with 18S rRNA.

Western blot and Immunoprecipitation (IP)

Cells were lysed by ice-cold lysis buffer [150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tri (pH 8.0) for 1 hr or subjected to nuclear and cytoplasmic extraction (NE-PER; Pierce Biotechnology, Rockford, IL). Lysates were then centrifuged at 13000 rpm for 10 min at 4°C to collect the supernate. Equivalent amounts of protein were separated on 4-12% gradient NuPAGE Bis-Tris Gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk containing 0.1% Tween20 for 1 hr at room temperature (RT), and incubated with primary antibodies overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase conjugated secondary antibodies. Enhanced chemiluminescence were used to detected target proteins. For IP, total 300 μg of lysate was incubated with the Dynabead Protein G (Invitrogen) -conjugated antibody at 4°C for 16 hr, and then subjected to a Western blot analysis. IgG was used as negative control. Rabbit polyclonal antibodies for total p27, p27 (S10A), total AKT and AKT (S473) were all purchased from Cell Signaling Technology (Danvers, MA, USA); Mouse monoclonal antibodies for Actin and Myc-tag were from Sigma-Aldrich (St. Louis, MO); monoclonal antibodies for Histone H1 and Tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, blocked in 5% bovine serum albumin, and incubated with primary antibody overnight at 4 °C. The cells were washed and incubated with Alexa Fluor 488-conjugated secondary antibodies for 1 hr at RT, followed by staining with 5 mg/mL 4', 6-diamidino-2-phenylindole before counting. Signals were examined using a fluorescence microscope.

Subcutaneous tumor models

All experimental procedures were approved by the Institutional Animal Care and Use Committee. 2×10^6 786-0 Con or KD cells were re-suspended, mixed with Matrigel (1□1 v/v) and then injected into 4-6 weeks

old athymic nude mice subcutaneously. Tumor-taken incidence was monitored and tumor volume was recorded weekly. At the end of experiment, animals were sacrificed and fresh tumor tissues were collected for further studies.

Bioinformatic and statistical analysis

The RNA-Sequencing-based mRNA expression data of DAB2IP gene in patients with ccRCC was all retrieved from The Cancer Genome Atlas (TCGA) which was open-access, and provided multidimensional genomic and clinical data [11]. For DAB2IP mRNA expression, 50% cut-off point was used to dichotomize the mRNA level as Low- and High- expression because they were molecules with no established cut-off points for their expression in ccRCC. The Kaplan-Meier analysis (long-rank test) was performed to analyze patient survival. Pearson's correlation coefficient was used to test the association between DAB2IP and p27 protein levels in ccRCC. Data from *in vitro* assay was presented as the mean ± SEM from three independent experiments, and the differences between two groups were compared by Student's two-tail t-test. All statistical analyses were performed by GraphPad Prism6 and SPSS16.0 software.

Results

DAB2IP is associated with decreased tumor size and better prognosis of ccRCC patients

DAB2IP is a known tumor suppressor in many types of cancer, it inhibits cell cycle progression and induces apoptosis [9, 12], however, its functions in RCC is unknown. By analysis of mRNA expression data of DAB2IP in 343 ccRCC patients from TCGA, we found that the patients with high DAB2IP mRNA level exhibit smaller tumor size than the patients with low DAB2IP (Fig 1 A). In addition, we examined the protein expression of DAB2IP using IHC in 90 ccRCC, and to be consistent, patients with high DAB2IP protein exhibit decreased tumor size compared to the patients with low DAB2IP (Fig 1 B). Moreover, the association of DAB2IP and survival of patients with ccRCC from TCGA was analyzed and the result indicated that patients with high DAB2IP showed increased overall survival (Fig 1 C), suggesting DAB2IP is associated with a better prognosis of ccRCC.

Cumulative evidence suggests that low expression of p27, a critical negative regulator of the cell cycle, is associated with tumor size and poor prognosis in patients with RCC[13-15], we therefore investigated the association of DAB2IP and p27 expression ccRCC samples, and found a positive correlation between them ($R= 0.42$, $P < 0.001$; Fig 1 D and E).

Loss of DAB2IP enhances RCC growth by suppressing p27

To further confirm the functional roles of DAB2IP in RCC cells and to clarify the potential relationship between DAB2IP and p27, HK-2 and 786-0 cells were transfected with either control shRNA (Con) or DAB2IP shRNA (KD), and then stable sublines were generated. Compared to Con cells, the expression of p27 protein in KD cells was significantly decreased, whereas, restoring of DAB2IP in KD cells could induce

p27 expression in a dose-independent manner (Fig 2 A). Along with the down-regulated p27 in KD cells, we observed promoted G1/S phase cell cycle progression and enhanced cell proliferation compared to Con cells (Fig 2 B and C). In addition, KD cells exhibited elevated growth ability *in vivo* and KD tumors showed decreased p27 as well as increased Ki-67 expression (Fig 2 D, E and F). Conversely, we noticed that overexpression of DAB2IP in RCC cells not only up-regulated p27 expression but also promoted TNF-α-induced cell growth inhibition (Supplemental Fig 1). Taken together, these data suggest that DAB2IP loss enhances RCC growth via suppressing p27 expression.

Loss of DAB2IP promotes p27 degradation in RCC cells

To further dissect the mechanisms of DAB2IP in regulating p27, we firstly checked the mRNA levels of p27 in Con and KD cells, and no significant difference was observed (Fig 3 A), which indicated that DAB2IP might not affect the gene transcription of p27. We then investigated whether DAB2IP was involved in the degradation or biosynthesis of p27 protein. Noticeably, although a proteasome inhibitor, MG132, increased the p27 proteins in both Con and KD cells, and the difference in p27 levels between Con and KD cells disappeared after the treatment of MG132 (Fig 3 B). When the protein biosynthesis in cells was blocked by using cycloheximide (CHX), we observed accelerated p27 degradation in KD cells compared to Con cells (Fig 3 C). Furthermore, the enhanced p27 degradation in KD cells was also confirmed by the increased p27 ubiquitination (Fig 3 D).

Loss of DAB2IP promotes p27 phosphorylation and cytosolic sequestration

Phosphorylation is considered an important mechanism for regulating p27 stability and/or localization and promoting its proteolysis [16, 17]. We found increased phosphorylation on Serine 10 (S10) of p27 protein in KD cells (Fig 4 A). p27 cytoplasmic localization is reported to be regulated by phosphorylation on S10 [18], fractionation of nuclear and cytoplasmic proteins demonstrated that p27 was mainly expressed in the cytosol of KD cells (Fig 4 B). Immunofluorescence (IF) staining also suggested that p27 was located in the nucleus of Con cells, but in KD cells, the majority of p27 was sequestrated in the cytosol (Fig 4 C). All these data indicated that loss of DAB2IP in RCC cells promotes p27 phosphorylation on S10 and cytosolic sequestration. To further confirm the effect of S10 phosphorylation on the function of p27, a mutant p27 was generated, in which the Serine was converted to Alanine at position 10 (S10A) and would escape from the phosphorylation on this site. Cell viability assay demonstrated that the introduction of p27 S10A cDNA significantly ablated the potentiated growth ability of KD cells (Fig 4 D and E).

AKT mediates the regulation of p27 by DAB2IP

PI3K/AKT signaling is the major regulation of p27 phosphorylation in RCC [17, 19]. We examined the activities of AKT in Con and KD cells, and found significant up-regulation of AKT phosphorylation (p-AKT, S473) in KD cells compared to Con cells, which indicated over-activation of AKT signaling in KD cells (Fig 5 A). PI3K/AKT specific inhibitor LY294002 significantly suppressed p-AKT, reduced phosphorylated p27 (S10) and induced total p27 expression (Fig 5 B). Our previous data showed the proline-rich domain in C

terminal (CPR) of DAB2IP directly interplayed with p85, the regulatory subunit of PI3K, and inhibited AKT activity in prostate cancer [9]. Herein, we demonstrated that both full-length (F) and CPR of the DAB2IP cDNA were able to suppressed AKT phosphorylation and induced p27 expression in KD cells (Fig 5 C).

To further confirm the critical role of AKT in mediating p27 phosphorylation on S10, 786-0 Con cells were transfected with constitutively activated AKT (ATK CA) combined with WT or S10A p27 cDNA. The results demonstrated that overexpression of AKT significantly induced phosphorylation of p27 on S10 and decreased total p27 expression. The total p27 level was only slightly increased in cells co-transfected with AKT and WT p27 compared to control (VC), however, in the cells co-transfected with AKT and S10A p27, total p27 level was dramatically increased (Fig 5 D). In addition, IP assay showed that AKT was able to interact with WT p27 but not S10A p27 (Supplemental Fig 2). These data indicated that AKT was failed to induce p27 phosphorylation and degradation when S10 was mutated in RCC cells. Moreover, consistent to the observation in Figure 4 E which showed that S10A p27 exhibited much more potency than WT p27 in cell growth suppression, S10A p27 was also more potent than WT p27 in ablating AKT-induced cell growth (Fig 5 E). Collectively, these data demonstrated that AKT was a critical mediator in the regulation of p27 by DAB2IP in RCC cells.

Discussion

DAB2IP gene encodes a novel Ras-GTPase activating protein (Ras-GAP) [20]. Profiles of this gene in organs show that it is highly expressed in normal prostate, brain, lung, bladder and kidney [20]. Subsequent studies demonstrate that DAB2IP is absent in many cancers mainly due to altered epigenetic regulation of its promoter [21–23]. Biological function studies of DAB2IP identify it as a tumor suppressor, which in normal tissues is essential for coordinating several cellular signaling pathways to maintain a homostasic condition [9, 12, 24]. Analysis of the amino acid sequence of human DAB2IP protein suggested that it contains several functional domains, including an pleckstrin homology (PH) domain with a high affinity for phosphoinositides, a C2 domain involved in binding phospholipids and a Ras-GAP domain in the N-terminal, and a period-like domain involved in binding to the intact RING finger of TNF-receptor-associated factor 2, a proline-rich (PR) domain involved in interacting with proteins containing an SH3 domain, and a leucine zipper domain in the C-terminal [3]. In prostate, DAB2IP facilitates TNF-α-induced apoptosis by dissociating apoptosis signal-regulating kinase 1 (ASK1) from its inhibitor via the PH and C2 domains, ablates aberrant cell proliferation via PR domain disturbing PI3K/AKT pathway [9, 25], and suppresses normal cell transformation via the Ras-GAP domain [12]. So far, the functions of DAB2IP in renal cell are not defined. In the present study, our data indicate that DAB2IP appears to function as a tumor suppressor in RCC and the mechanism of action is mediated by p27.

As uncontrolled cellular proliferation is the main hallmark of cancer, alterations in the expression or activity of proteins which are intimately involved in cell cycle regulation are of particular interest as novel putative biological markers. p27 is a negative regulator of the G1 phase of the cell cycle, is regarded as a tumor suppressor gene, and is frequently lost in tumor cells [26]. This protein is frequently deregulated in

RCC and its clinical significance has been well clarified. Tsuneyoshi's study [13] shows that low expression of p27 is associated with tumor size and poor prognosis in patients with RCC. In Chkhotua's study [14], loss of p27 is a predictor of poor recurrence-free and cancer-specific survival in RCC. When the sub-cellular location of p27 is stratified, loss of nuclear p27 is a frequent event in human RCC and is a powerful predictor of poor outcome [15], while cytoplasmic p27 is reported to elevate in tumors compared with matched controls [17]. Cytoplasmic mislocalization of p27 is associated with increasing tumor grade, and knockdown of p27 or relocalization to the nucleus increases apoptosis in RCC cells [17].

Nevertheless, the molecular mechanisms about how p27 is regulated in RCC are less well-defined. PI3K/AKT mediated mTORC1 or mTORC2 signaling pathways appear to be the predominant regulators for p27 phosphorylation and cellular localization in RCC cells [17, 19]. We demonstrate that loss of DAB2IP is able to activate AKT, contributing to p27 phosphorylation and cytoplasmic sequestration. There are multiple phosphorylation sites on p27 protein. Walker [17] demonstrates that AKT phosphorylates p27 on T157 but the status of S10 is not determined in RCC cells. Our and Meloche's data [18] suggest AKT is also able to phosphorylate p27 on S10 and the phosphorylation of S10 is necessary for its nuclear to cytoplasmic redistribution. In addition, cyclin-dependent kinase (CDK)-dependent phosphorylation of p27 on T187 is required for its degradation, which is mediated by the SKP2 ubiquitin-proteasome pathway [27]. In RCC specimens, the expression of p27 is inversely correlated with SKP2 [28]. Our previous data indicate that DAB2IP can suppress SKP2 expression in prostate cancer [29], and in RCC, we find SKP2 is up-regulated in DAB2IP KD cells compared to Con cells (data not shown). Although further study is needed to determine the role of SKP2 in DAB2IP-regulated p27 in RCC, all our findings suggest that DAB2IP is essential for p27 stabilization in RCC, which is at less partly mediated by PI3K/AKT pathway.

Declarations

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Author contributions KW and WQ conceived the project; JZ designed the experiments, analyzed the data, and wrote the manuscript; LW and XP performed most of the experiments and analyzed the data; ZD carried out the animal works and Bioinformatic data mining. All authors have read and agreed to the published version of the manuscript and declare no competing financial interests.

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

Conflict of interest The authors have no potential confict of interest to declare.

Ethical approval This study was approved by with the Ethics Committees at Shaanxi Provincial People's Hospital. The written informed consents were acquired from the guardians of all donors and the animal care and experimental procedures were approved by the Animal Experimental Committee of Hospital.

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Figures

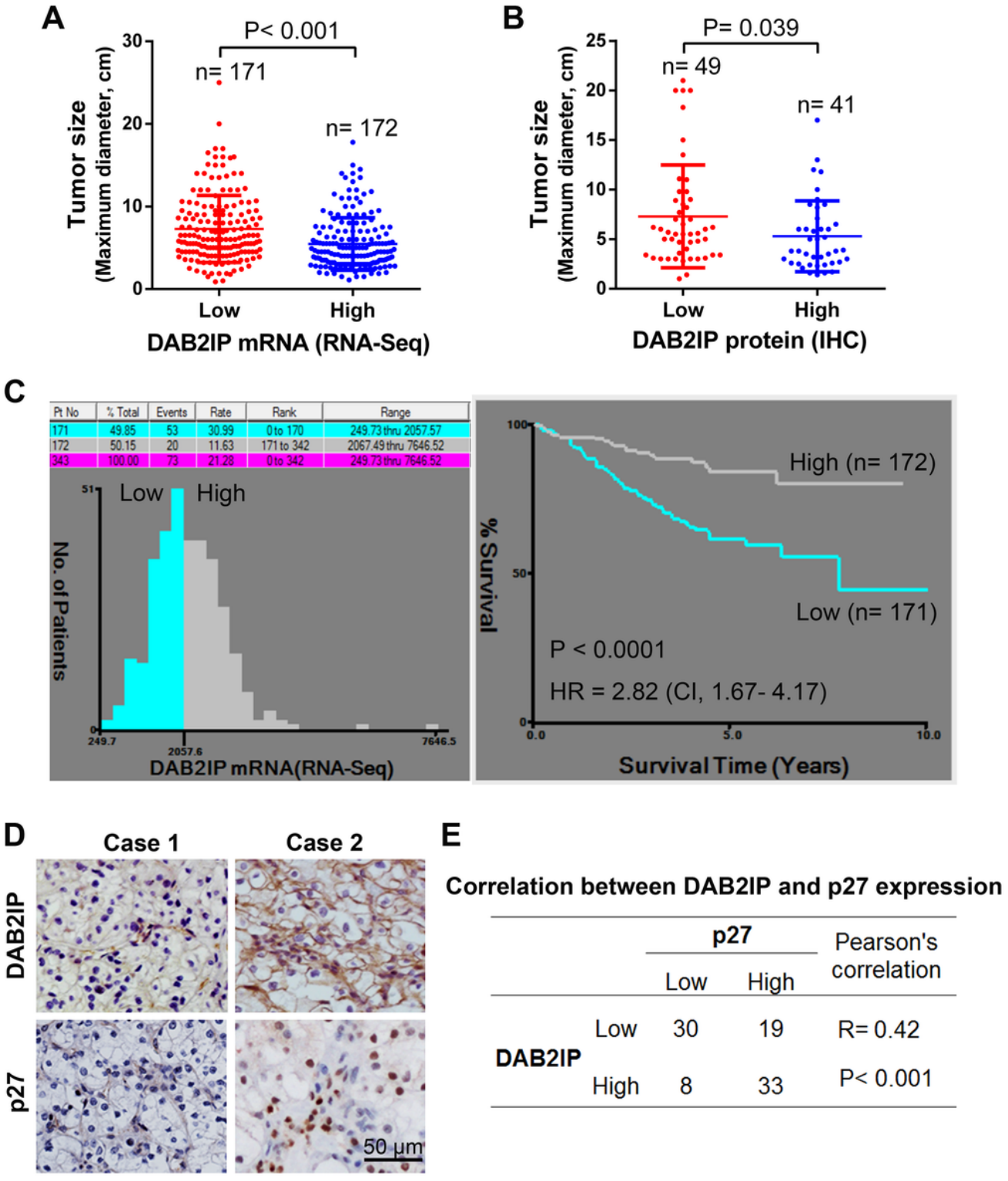

Figure 1

DAB2IP is associated with decreased tumor size and better prognosis of ccRCC patients. (A) Association between DAB2IP mRNA level (RNA-Sequencing) and tumor size of ccRCC from TCGA. (B) Association between DAB2IP protein level (IHC) and tumor size of ccRCC. (C) Left, 50% cut-off point was used to dichotomize the DAB2IP mRNA level as Low- and High- expression; Right, Kaplan-Meier analysis (long-rank test) of the association between DAB2IP level and overall survival of ccRCC from TCGA. (D)

Representative IHC staining of DAB2IP and p27 in ccRCC samples. (E) Pearson's correlation analysis of DAB2IP and p27 protein levels in ccRCC.

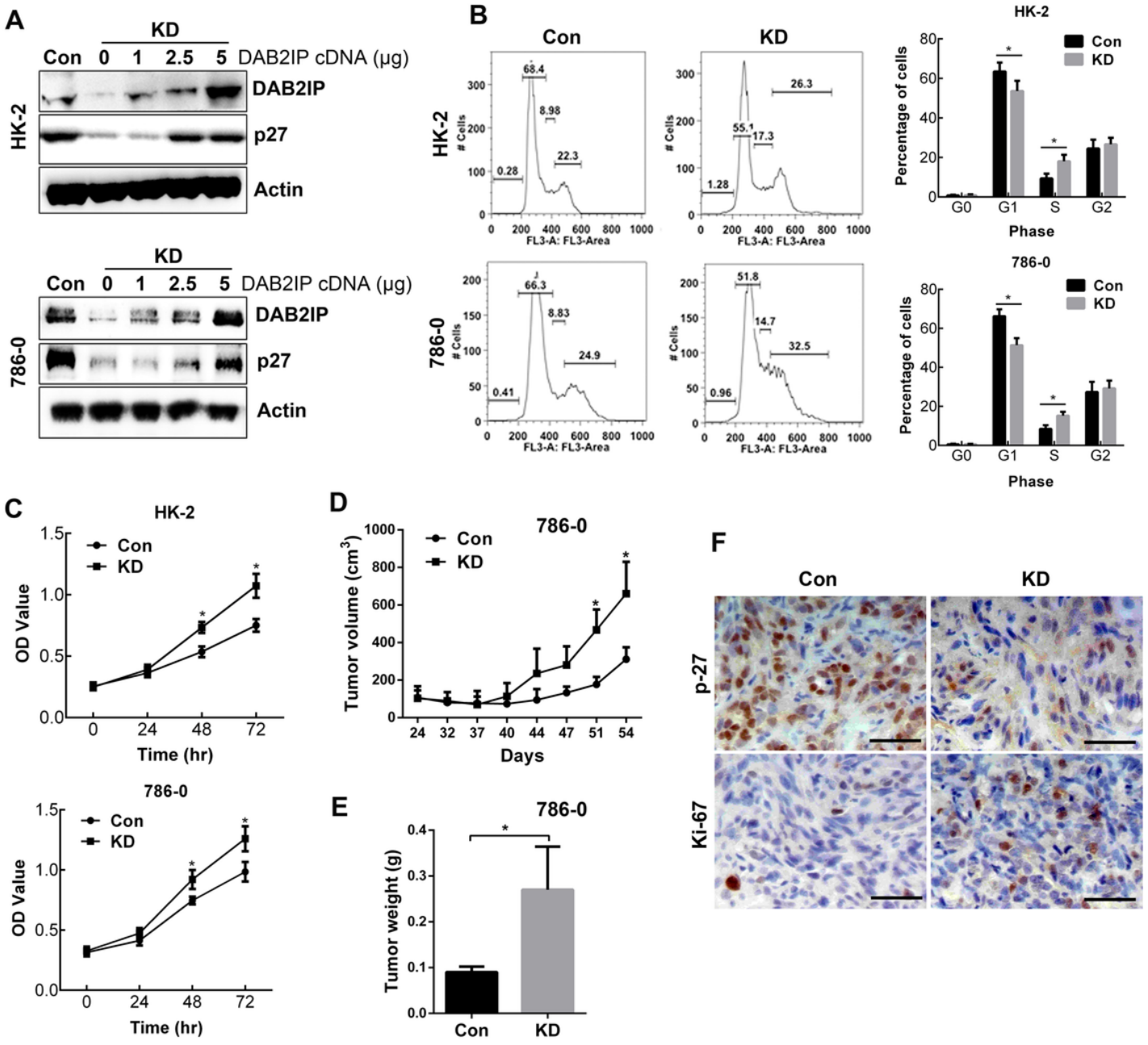

Figure 2

Loss of DAB2IP enhances RCC growth by suppressing p27. (A) Western blot of control (Con), DAB2IP knockdown (KD) cells and KD cells transfected with different amount of DAB2IP cDNA. (B) Cell cycle assay of HK-2 Con and KD cells, 786-0 Con and KD cells. (C) Cell proliferation of Con and KD cells cultured in 0.5% FBS medium. Subcutaneous xenografts 786-0 Con and KD cells were established, tumor growth curve (D) and tumor weight at the end of experiment (E) were shown. (F) IHC staining of p27 and Ki-67 in 786-0 Con and KD tumor tissues. Scale bar, 200 μm; *, $P < 0.05$.

Figure 3

Loss of DAB2IP promotes p27 degradation. (A) Real-time PCR detecting p27 mRNA expression in Con and KD cells. ns, not significant. (B) Western blot of p27 expression in Con and KD cells with or without MG132 treatment (10 μM, 9 hr). (C) Con and KD cells were pretreated with serum-free medium for 2 hr, followed by the CHX (7.5ug/ml) treatment. After the indicated time of CHX treatment, the expression of p27 in cells was determined by Western blot. Quantification of p27 protein levels were shown. (D) IP detecting of the ubiquitin-binding p27 (Ub) in 786-0 Con and KD cells.

Figure 4

Loss of DAB2IP promotes p27 phosphorylation and cytosolic localization. (A) Western blot of total p27, phosphorylated p27 (p-p27, S10) in Con and KD cells. (B) Western blot of p27 expression in nuclear and cytoplasmic fractions of Con and KD cells. (C) IF detecting the localization of p27 in 786-0 Con and KD cells. 786-0 KD cells were transfected with control vector (VC), wildtype (WT) or mutant (S10A) p27 constructs, protein expression (D) and cell viabilities (E) of the indicated cells were then determined. *, P < 0.05.

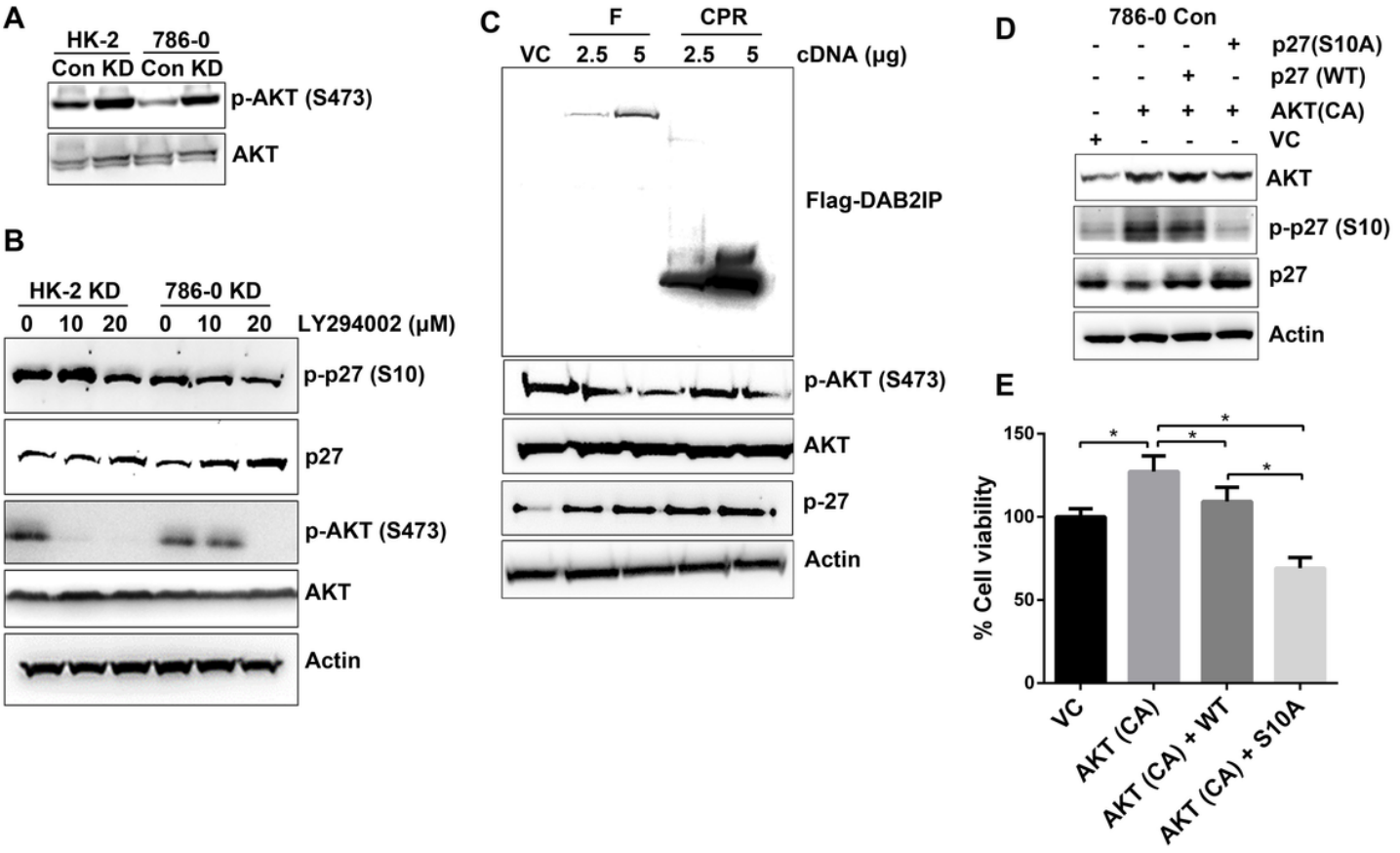

Figure 5

AKT mediates the regulation of p27 by DAB2IP. (A) Western blot of total and phosphorylated AKT in Con and KD cells. (B) HK-2 KD and 786-0 KD cells were treated with PI3K/AKT pathway inhibitor LY294002 for 24 hr, then total and phosphorylated AKT and p27 were determined. (C) 786-0 KD cells were either transfected with VC, DAB2IP full-length cDNA (F) or truncate cDNA coding the CPR domain of DAB2IP (CPR), then Flag-tag antibody was used to detect the exogenous DAB2IP expression, and the expression of AKT and p27 in cells were determined by Western blot. 786-0 Con cells were either transfected with VC, Constitutively active AKT (AKT CA), WT or mutant (S10A) p27 constructs, then protein expression (D) and cell viabilities (E) in the indicated cells were determined. *, $P < 0.05$.

Supplementary Files

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- Supplementaryfigures.docx