

Original Article

Tob1 is low-expressed in adrenocortical carcinoma and regulates steroidogenic enzymes expression and cell apoptosis of human adrenal cortical cell

Xiaoliang Li¹, Ruihua Lun², Lizhen He¹, Hongbin Liu¹

¹Department of Laboratory, The First Affiliated Hospital of Henan Polytechnic University, The Second People's Hospital of Jiaozuo, Jiaozuo 454000, Henan, China; ²Department of Laboratory, Jiaozuo Maternal and Children's Hospital, Jiaozuo 454000, Henan, China

Received July 27, 2017; Accepted April 12, 2018; Epub June 15, 2018; Published June 30, 2018

Abstract: Background: Adrenocortical carcinoma (ACC) is an uncommon endocrine malignancy tumor. Finding an effective method is still needed to help diagnose and treat ACC. The aim of the present study was to evaluate the potential effect of Tob1 on ACC. Methods: Tob1 expression in adrenal tissues of patients with ACC or adrenocortical adenoma (ACA) was detected by immunohistochemical staining, western blotting and RT-PCR, respectively. For the *in vitro* assay, Tob1 overexpression plasmid (pcDNA3.0/Tob1) or empty plasmid (pcDNA3.0) was transfected into H295R cells. The cortisol concentration was detected by ELISA. The mRNA levels of steroidogenic enzymes including CYP11A1, CYP17A1, and 3 β -HSD were analyzed by RT-PCR. The cell apoptosis of H295R cells was analyzed by FCM. The expression of k-Ras, p-Raf, Raf, p-MEK, MEK, p-ERK, and ERK were detected by western blotting. Results: Protein and mRNA levels of Tob1 in ACC were significantly decreased as compared with control group. The mRNA levels of CYP11A1, CYP17A1, and 3 β -HSD was inhibited by Tob1 overexpression. The cortisol concentration in culture supernatant of H295R cells was also reduced by Tob1 overexpression. Tob1 overexpression induced cell apoptosis of H295R cells. In addition, Tob1 reduced the phosphorylation of Raf, MEK and ERK, suggesting that Ras-Raf-MEK-ERK pathway was involved in the effect of Tob1 on H295R cells. Conclusion: The results indicated that low-expressed Tob1 might be useful to differentiate between ACC and ACA clinically. In addition, Tob1 is a tumor suppressor of ACC and provided a new insight in ACC treatment.

Keywords: Adrenocortical carcinoma (ACC), Tob1, Ras/Raf/MEK/ERK pathway, steroidogenic enzymes, cortisol secretion

Introduction

Adrenocortical carcinoma (ACC) is an uncommon endocrine malignant tumor and has an incidence ranging from 0.7 to 2.0 cases/million per year [1, 2]. Approximately 30-70% of the patients present with advanced ACC didn't have effective therapeutic options [3]. Mitotane is the only FDA-approved drug for ACC. However, the usage of mitotane should be carefully considered and monitored because of the narrow therapeutic window and severe adverse effects [3]. As of today, chemotherapy remains the standard treatment to locally advanced or metastatic ACC. The pathologic diagnosis of ACC is based on multiple morphologic parameters that are suggestive but not pathognomonic of

malignancy [4-6]. Molecular analyses have been recently attempted in helping diagnose ACC in recent years. Although hypermethylation, miRNA, TP53, ZNRF3, β -catenin are proved to be possible candidates to help diagnose and establish prognosis, they are not yet used in clinical pathologic diagnosis for ACC [7].

Transducer of erbB2.1 (Tob1) is a member of the B-cell translocation gene (BTG)/transducer of erbB2 (Tob) anti-proliferative protein family [8, 9]. Accumulated evidences confirm that Tob1 is involved in the negative regulation of cell growth and functions as a tumor suppressor in several malignancies [10, 11]. It has been proved that Tob1 is low-expressed or lost in a variety of cancers including hepatocellular car-

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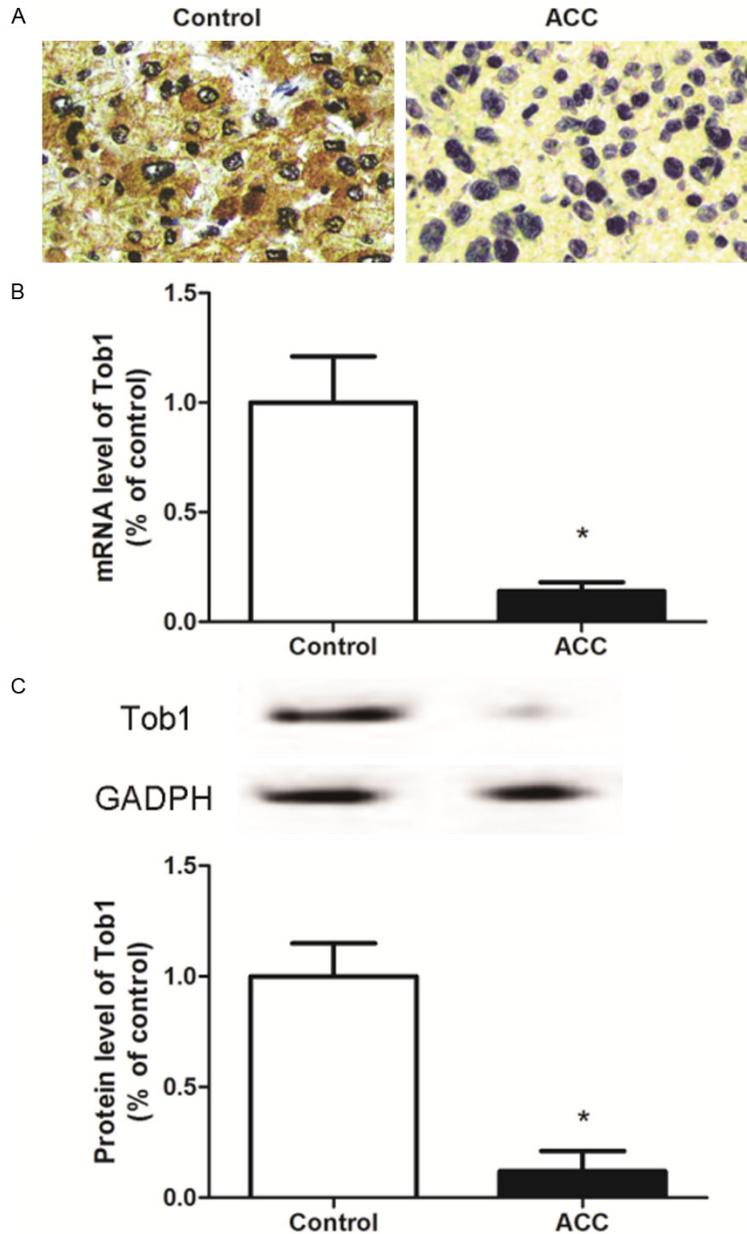


Figure 1. Tob1 was low-expressed in ACC. Tob1 expression in adrenal tissues of patients with ACC or ACA was detected by immunohistochemical staining, western blotting and RT-PCR. A. Immunohistochemical staining ($\times 100$); B. RT-PCR; C. Western blotting. Control group: patients with ACA; ACC group: patients with ACA. * $P < 0.05$ vs. control group.

cinomas, lung cancer, breast and pancreas [12]. However, the role of Tob1 in ACC is still unknown.

The Ras-Raf-MEK-ERK mitogen activated protein kinase (MAPK) pathway signals from cell surface receptors to transcription factors, and regulates multiple gene expression [13]. The pathway has diverse effects in various physiological processes as diverse as cell cycle pro-

gression, cell apoptosis and cell differentiation [14]. Besides its established role in physiological processes, Ras-Raf-MEK-ERK pathway has been shown to play key roles in tumorigenesis [13]. It has been proved that abnormal activation of Ras-Raf-MEK-ERK pathway occurs in several human cancers due to the mutation of upstream molecules [13]. Whether the pathway is normal in ACC remains unknown.

The aim of the present study was to investigate the role of Tob1 in ACC. The results proved that Tob1 was low-expressed in patients with ACC. Tob1 overexpression induced cell apoptosis of H295R and influenced regulation of steroidogenesis via inhibiting Ras-Raf-MEK-ERK pathway. The results indicated that low-expression of Tob1 can be used to distinguish ACC from ACA in clinical pathologic diagnosis. Besides, Tob1 is likely to be a promising molecular therapy target for ACC treatment.

Material and methods

Patients

Adrenal tissue samples were obtained from patients with ACC (n=7) and adrenocortical adenoma (ACA, n=4). Pathological diagnosis was carried out by expert pathologists, which was based on Weiss's criteria [15]. A score of ≥ 3 was

considered as ACC. Informed consent was obtained from each patient. The present study was approved by the medical ethics committee of our hospital.

Immunohistochemical staining

The fixed tissues were serial sectioned at 5 μ m thickness. To evaluate the expression of Tob1, immunohistochemical staining was performed

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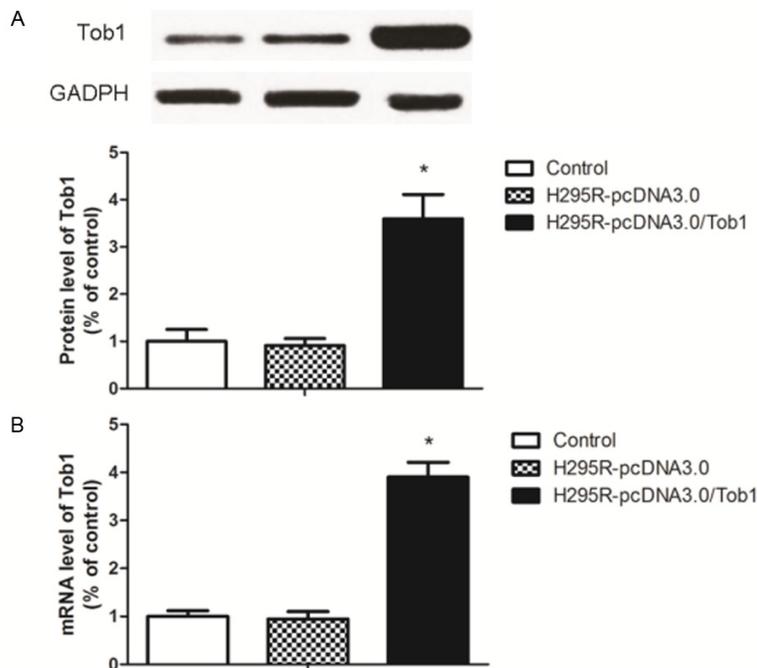


Figure 2. Tob1 was over-expressed in H295R-pcDNA3.0/Tob1 cells. Tob1 overexpression plasmid (pcDNA3.0/Tob1) or empty plasmid (pcDNA3.0) was transfected into H295R cells. Protein and mRNA levels of Tob1 in H295R cells were detected by western blotting and RT-PCR, respectively. A. Western blotting; B. RT-PCR. Control group: normal H295R cells; H295R-pcDNA3.0 group: H295R cells transfected with pcDNA3.0 plasmid; H295R-pcDNA3.0/Tob1 group: H295R cells transfected with pcDNA3.0/Tob1 plasmid. *P<0.05 vs. H295R-pcDNA3.0 group.

using a commercial kit (Fuzhou Maixin Biotechnology Co., Ltd., Fuzhou, China) according to manufacturer's instructions. The antibody against Tob1 (Santa Cruz Biotech, Santa Cruz, CA, USA) were diluted in PBS (1:1000).

Quantitative RT-PCR

The adrenal tissues were stored in liquid nitrogen and then homogenized with liquid nitrogen for RNA extraction. Total RNA was extracted from homogenized adrenal tissues and H295R cells using Trizol reagent (Invitrogen, CA, USA) according to the manufacture's recommendations. RNA quality was detected by agarose gel electrophoresis. And then the total RNA was reverse transcribed to cDNA using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Shiga, Japan). Real-time PCR was carried out using SYBR Green PCR Master Mix (Thermo Scientific, Waltham, MA, USA). PCR cycles were performed on a real-time quantitative PCR instrument (Bio-Rad) with the following conditions: denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at

95°C for 15 s, annealing at 54°C for 10 s and extension at 72°C for 30 s. The primers of Tob1 and GADPH were synthesized by Sangon (Shanghai, China). Tob1 forward primer: 5'-CTTC AGGA GGTC GTTC AC-AT-3', reverse primer: 5'-AACC TTTA CCAC TGCC ACTT-3'; CYP11A1 forward primer: 5'-CAGA CGCA TCAA GCAG CAA-3', reverse primer: 5'-CTGG AGGC AGGT TGAG CAT-3'; CYP17A1 forward primer: 5'-TCTG GGCA CTGC ATCA CG-3', reverse primer: 5'-GCTC CGAA GGGC AA-AT AACT-3'; 3β-HSD forward primer: 5'-GCTT CCAA ACGC TGAC ACCA-3', reverse primer: 5'-GGCC CTGT GATC CATC CA-AT AG-3'; GADPH forward primer: 5'-AGCC ACAT CGCT GA-GA CA-3', reverse primer: 5'-GCCC AATA CGAC CAAA TCC-3'. Relative quantification was calculated as fold changes according to the $2^{-\Delta\Delta Ct}$ method. GADPH was used as the endogenous control.

Cell culture and treatment

Human adrenal cortical cell (NCI-H295R cell) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Culture of H295R cells was performed as described previously [16]. The Tob1 overexpression plasmid was constructed by inserting the cDNA fragment of Tob1 into pcDNA3.0 plasmid. The cDNA fragment was retro-transcribed from the full-length of Tob1 mRNA. H295R cells were transfected with Tob1 overexpression plasmid (pcDNA3.0/Tob1) or empty plasmid (pcDNA3.0) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). Stable expression clones were selected by G418 (400 μg/mL, BD Biosciences Clontech, CA, USA).

Western blot analysis

The homogenized adrenal tissues and H295R cells were lysed in RIPA lysis buffer. Proteins were separated by SDS-PAGE on a 12% gel and transferred to a nitrocellulose membrane (Amersham Pharmacia, Germany). The membranes

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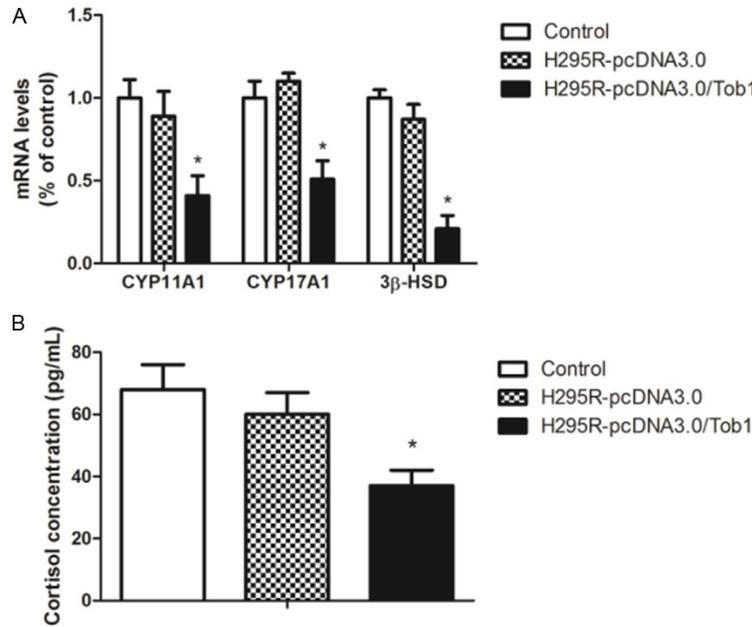


Figure 3. Tob1 inhibited cortisol secretion and steroidogenic enzymes expression. The cortisol concentration in culture supernatant was detected by ELISA. The mRNA levels of steroidogenic enzymes including CYP11A1, CYP17A1, and 3β-HSD were analyzed by RT-PCR. A. RT-PCR; B. ELISA. Control group: normal H295R cells; H295R-pcDNA3.0 group: H295R cells transfected with pcDNA3.0 plasmid; H295R-pcDNA3.0/Tob1 group: H295R cells transfected with pcDNA3.0/Tob1 plasmid. * $P < 0.05$ vs. H295R-pcDNA3.0 group.

were blocked in 5% (w/v) powdered low fat skim milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 2h. Then the membranes were incubated with anti-Tob1, anti-k-Ras, anti-p-Raf, anti-Raf, anti-p-MEK, anti-MEK, anti-p-ERK, anti-ERK, and anti-GADPH antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA, 1:1000 dilution in TBST) at 4°C for 8 h, and then blotted with peroxidase secondary antibody (Santa Cruz Biotech, 1:2000 dilution in TBST) at 37°C for 1 h. Chemiluminescent detection was performed using Bio-Rad ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). Gray value of the bands was analyzed by Image J2x software.

Cortisol detection

Cortisol level in culture supernatant was detected using an ELISA kit (R&D Systems, Inc.) in accordance with manufacturer's instructions. Briefly, the microtiter plates used in the assay were coated with purified antibody. The standards or samples were added into the plates, and then anti-cortisol antibody and

horseradish peroxidase (HRP)-labeled avidin were subsequently added. The 3,3',5,5'-tetramethylbenzidine was used as the substrate of HRP for color reaction. Finally, the optical density (OD) value was read using a microplate reader (Tecan Sunrise basic, Groedig, Austria) at the wavelength of 450 nm. Cortisol levels were calculated using a standard curve.

Cell apoptosis assay

Cell apoptosis of H295R cells was performed using an Annexin V-PI Apoptosis Detection Kit (Abcam, Cambridge, UK) according to the the manufacturer's recommendations. Briefly, H295R cells were trypsinized, washed and suspended in 500 μL of binding buffer. Then the cells were incubated with Annexin V-FITC and Propidium Iodide (PI). Cells were incubated for 15 min avoiding

light. The relative quantitative apoptosis was analyzed by flow cytometry (Beckman Coulter).

Statistical analysis

All values are expressed as means ± SD of three independent experiments. Statistical analysis was performed using SPSS version 19.0 software. The significance of differences was evaluated by One-way repeated measures analysis of variance (ANOVA). Differences with a p value less than 0.05 were considered statistically significant.

Results

Tob1 is low-expressed in ACC

To evaluate the Tob1 expression and its role in ACC, protein and mRNA expression levels of Tob1 in adrenal tissues were detected by western blotting, immunohistochemical staining and RT-PCR. As shown in **Figure 1A**, the staining of positive cells in control group (patients with ACA) was stronger than ACC group (patients with ACC). In addition, mRNA level of Tob1 in

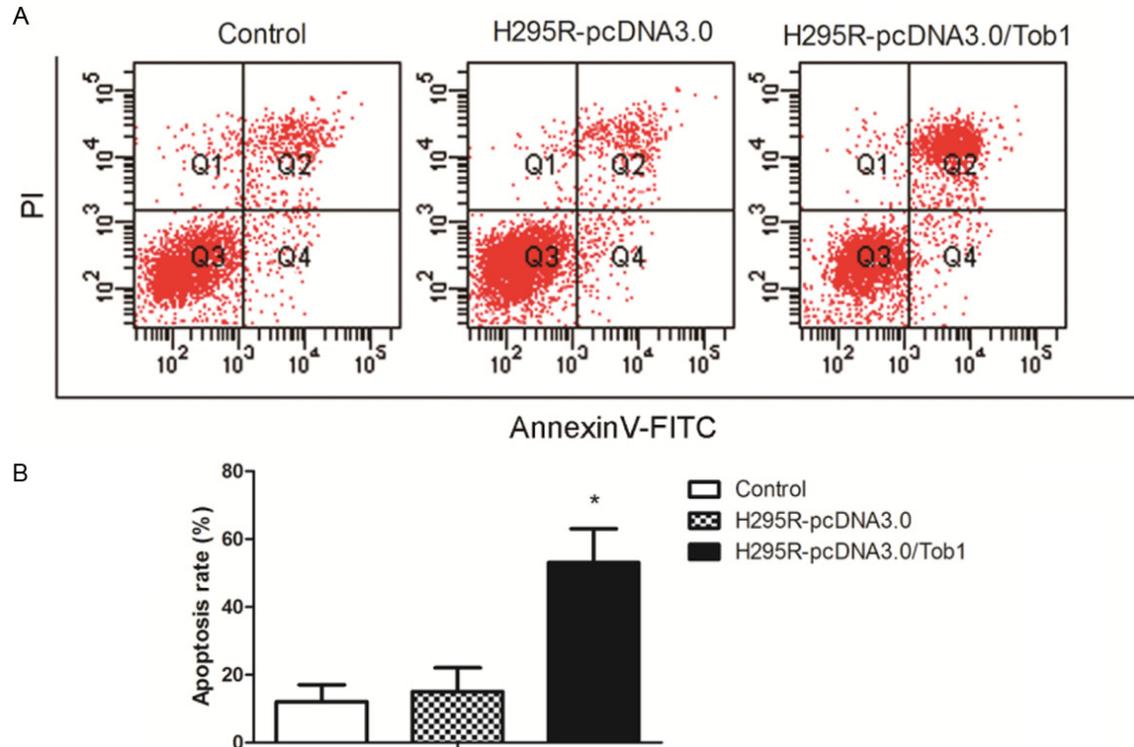


Figure 4. Tob1 induced cell apoptosis of H295R cells. The cell apoptosis of H295R cells was analyzed by flow cytometry method. A. Cell apoptosis of H295R cells with different treatment; B. Quantitative analysis of cell apoptosis. Control group: normal H295R cells; H295R-pcDNA3.0 group: H295R cells transfected with pcDNA3.0 plasmid; H295R-pcDNA3.0/Tob1 group: H295R cells transfected with pcDNA3.0/Tob1 plasmid. *P<0.05 vs. H295R-pcDNA3.0 group.

ACC group was significantly decreased compared with control group (Figure 1B). And the protein level of Tob1 exhibited a significant reduction in ACC group (Figure 1C). The results indicated that low-expression of Tob1 can be used to distinguish ACC from ACA in clinical pathologic diagnosis.

Tob1 is over-expressed in H295R-pcDNA3.0/Tob1 cells

As we presented that Tob1 was low-expressed in ACC, then the role of Tob1 in ACC was also evaluated. The H295R cells were transfected with empty vector (pcDNA3.0) or pcDNA3.0/Tob1. The mRNA and protein levels of Tob1 were detected by RT-PCR and western blotting, respectively. As shown in Figure 2, the protein and mRNA levels of Tob1 in pcDNA3.0/Tob1 transfected H295R cells (H295R-pcDNA3.0/Tob1 cells) were increased by 2.96- and 3.11-fold, compared with empty pcDNA3.0 transfected H295R cells (H295R-pcDNA3.0 cells).

Tob1 inhibites cortisol secretion and steroidogenic enzymes expression

It has been proved that about 40-60% of patients with malignant tumors of the adrenal glands exhibited excessive hormonal production, such as hypercortisolism or hyperandrogenism [17, 18]. The cortisol concentration was detected by ELISA. The mRNA levels of steroidogenic enzymes including CYP11A1, CYP17A1, and 3β-HSD were analyzed by RT-PCR. As shown in Figure 3A, the mRNA levels of CYP11A1, CYP17A1, and 3β-HSD were inhibited by Tob1 overexpression. The cortisol concentration in culture supernatant of H295R cells was also reduced by Tob1 overexpression (Figure 3B).

Tob1 induces cell apoptosis of H295R cells

To investigate the effect of Tob1 on cell apoptosis of H295R cells, FCM was carried out. The results in Figure 4 showed that cell apoptosis in

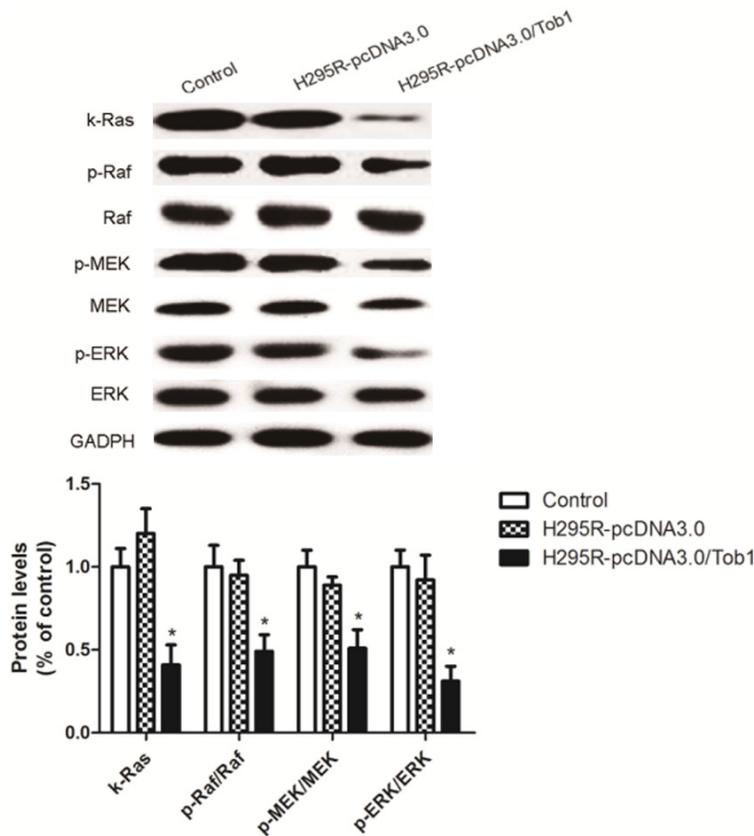


Figure 5. Ras-Raf-MEK-ERK pathway was involved in the effect of Tob1 on H295R cells. The expression of k-Ras, p-Raf, Raf, p-MEK, MEK, p-ERK, and ERK was detected by western blotting. Control group: normal H295R cells; H295R-pcDNA3.0 group: H295R cells transfected with pcDNA3.0 plasmid; H295R-pcDNA3.0/Tob1 group: H295R cells transfected with pcDNA3.0/Tob1 plasmid. *P<0.05 vs. H295R-pcDNA3.0 group.

H295R-pcDNA3.0/Tob1 cells was significantly increased by 2.53-fold as compared to the H295R-pcDNA3.0 cells. The data indicated that Tob1 overexpression induced cell apoptosis of H295R cells.

3.5 Ras-Raf-MEK-ERK pathway is involved in the effect of Tob1 on H295R cells

Previous studies proved that abnormal activation of Ras-Raf-MEK-ERK pathway usually occurs in several human cancers. To clarify whether Tob1 regulated H295R cells by Ras-Raf-MEK-ERK pathway, the expression of k-Ras, p-Raf, Raf, p-MEK, MEK, p-ERK, and ERK were detected by western blotting. As shown in **Figure 5**, the expression of k-Ras, p-Raf, p-MEK and p-ERK were decreased by Tob1 overexpression, indicating that Tob1 reduced the phosphorylation of Raf, MEK and ERK. The results suggested that Ras-Raf-MEK-ERK pathway was

involved in the effect of Tob1 on H295R cells.

Discussion

Since ACC is an endocrine malignancy with poor prognosis, a more effective diagnostic and therapeutic approach is needed. Numerous studies proved that Tob1 played an important role in malignant tumors [19, 20]. Tob1 expression was down-regulated in various cancers, including particular breast cancer, pancreas cancer, thyroid cancer, and stomach cancer [20]. In addition, it has been reported that Tob1 expression was either abolished or reduced in 75% of patients with gastric cancer [21]. In the present study, the Tob1 expression in ACC and ACA was detected, the results showed that Tob1 was low-expressed in adrenal tissues of patients with ACC, suggesting Tob1 might serve as a diagnostic marker for ACC.

It has been proved that malignant tumors of the adrenal glands usually exhibit abnormal hormonal production [7]. IFN- β possesses anti-tumor effect and has been reported to inhibit cell growth and cortisol secretion in human adrenocortical carcinoma cells [22]. IFN- β also inhibited the expression of steroidogenic enzymes including CYP11A1, CYP17A1 and CYP11B1 [22]. It has been reported that tumor suppressor inhibits tumor growth by blocking cell cycle and/or inducing cell apoptosis [19]. Tob1 is a tumor suppressor and inhibits cell proliferation, blocks cell cycle and induces cell apoptosis [20, 23]. Besides, Tob1 decreases the migration, invasion, and metastatic ability of cancer cells [20]. In the present study, Tob1 overexpression plasmid was transfected into H295R cells. We found that Tob1 overexpression inhibited cortisol secretion and steroidogenic enzymes expression. Tob1 also induced cell apoptosis of H295R cells. These results indicated that Tob1 might be a tumor suppressor of ACC.

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Ras is a small GTP-binding protein, which is the common upstream molecule of several signaling pathways including Raf/MEK/ERK, PI3K/Akt and RalEGF/Ral [24]. Previous published studies have proved that Tob1 suppressed cancers via many pathways, such as MAPK and Wnt/ β -catenin [25, 26]. Our pretest study proved that Raf/MEK/ERK was influenced by Tob1 overexpression. Abnormal activation of Ras-Raf-MEK-ERK pathway occurs in several human cancers. Besides regulation of cell proliferation and cell apoptosis, Ras-Raf-MEK-ERK pathway also plays an important role in cell growth, malignant transformation and drug resistance [13]. In the present study, Tob1 overexpression suppressed the expression of k-Ras, and inhibited the phosphorylation of Raf, MEK and ERK. The results suggested that Ras-Raf-MEK-ERK pathway was involved in the effect of Tob1 on H295R cells.

Conclusions

In summary, the present study evaluated the potential effect of Tob1 in ACC. The results showed that protein and mRNA levels of Tob1 in ACC were significantly decreased. Tob1 overexpression inhibited cortisol secretion, decreased steroidogenic enzymes expression and induced cell apoptosis of H295R cells. In addition, Ras-Raf-MEK-ERK pathway was involved in the effect of Tob1 on H295R cells. The results proved that Tob1 is a tumor suppressor of ACC and can be helpful for ACC diagnose. The results indicated that Tob1 can be used to distinguish ACC from ACA clinically and targeted therapy of ACC

Disclosure of conflict of interest

None.

Address correspondence to: Xiaoliang Li, Department of Laboratory, The First Affiliated Hospital of Henan Polytechnic University, The Second People's Hospital of Jiaozuo, Jiaozuo 454000, Henan, China. Tel: +86 03912758507; E-mail: lixl0721@126.com

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