BATF Inhibits Cell Proliferation and Migration via PI3K/AKT/mTOR Pathway in Clear Cell Renal Cell Carcinoma

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Research Article

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Abstract

Background

Previous studies reported that BATF played an important role in the progression of various cancers, but no report had been found on clear cell renal cell carcinoma(ccRCC). So we investigated the effects of BATF on the progression of ccRCC.

Methods

In this study, using TCGA-KIRC database from the Cancer Genome Atlas to analyze the differential genes of BATF in ccRCC ; using immunohistochemistry to detect BATF expression in 75 ccRCC tumorous and 28 nontumorous tissues, and investigate its relationship with clinicopathological parameters. Moreover, we constructed the cell lines of BATF overexpression and knockout in Caki-1and 786-0 ccRCC cell lines and confirmed by western blot. CCK8 assay was used to evaluated the cell viability and using EdU staining to detect cell proliferation; using Transwell experiment to investigate the affection of BATF in ccRCC cell migration. Westernblot was used to detect the phosphorylation of PI3K/AKT/mTOR.

Results

These results revealed that BATF expression in ccRCC tumorous was significantly higher compared with that in adjacent nontumorous tissues and was significantly correlated with T stage, Fuhrman grade, Tumor necrosis and status. In addiditon, BATF overexpression significantly inhibited the viability, proliferation and migration of the two cell lines. More importantly, using westernblot showed that silencing BATF significantly increased the level of phosphorylated AKT, PI3K and mTOR, while BATF overexpression decreased phosphorylation that involved in the PI3K/Akt/mTOR signaling pathway.

Conclusions

Our findings demonstrate that BATF plays an important role in the progression of ccRCC and it may act as an tumor supressor gene to inhibit the progression of ccRCC through regulating the PI3K/Akt/mTOR signaling pathway.

1. Introduction

Renal cell cancer(RCC) is one of the most common malignancies in the world originating from the renal parenchymal urinary tubular epithelial system. The incidence of RCC in China is increasing at a speed of about 2.5% per year[1]. However, the 5-year survival rate of metastatic RCC is less than 10% because of its poor prognosis due to its insensitivity to radiotherapy and chemotherapy and even about 30% of localized RCC has local recurrence after operation[2]. RCC has many pathological types and clear cell

renal cell carcinoma (ccRCC) is the most common type, accounting for 80% of all RCC[3]. Although RCC treatments have been improved, but still cannot achieve satisfactory prognosis, most patients die in short time because of relapse and metastasis. So far, the mechanism is still unclear for ccRCC.

With the development of bioinformatics, B cell activating transcription factor (BATF) has been found to be closely related to the proliferation and migration of ccRCC cells. BATF is a family member of Alkaline Leucine Zipper Transcription Factor Activator Protein 1(AP-1)[4]. AP-1 is considered as a kind of intracellular transcription activator, mainly composed of proto-oncogene encoding protein Jun and Fos, which binds DNA target sequence in the form of homodimer or heterodimer complex, regulating the expression of target genes. BATF form heterodimer with Jun-B protein,which control transcription of target gene.

At present, the therapeutic agents targeting VEGF or mTOR signaling, such as Pazopanib, Sunitinib, Temsirolimus, Bevacizumab and Interferon a-2b5, have been successfully clinically useful[5]. However, these drugs only produce a partial improvement for the prognosis of ccRCC and the efficacy for advanced ccRCC are still disappointing. BATF targeting effector molecules are widely distributed, including a large number of transcription factors, T cell activation related molecules, cytokine signaling pathway molecules, apoptotic process molecules, cell migration related molecules and CD8 + T cell effector molecules[6]. By exploring the molecular regulation mechanism of BATF in renal cell carcinoma, we can provide new ideas and strategies for clinical research of ccRCC.

2. Materials And Methods

2.1 BATF in TCGA-KIRC database.

GEO is a public functional genomics data repository.The data of BATF expression in ccRCC and matched adjacent normal tissue was downloaded from TCGA-KIRC (The Cancer Genome Atlas-Kidney Renal Clear Cell Carcinoma) through this network (https://portal.gdc.cancer.gov/ and https://dcc.icgc.org/releases/release_27/Projects) to analyze the differential expression of BATF in different grades and stages of ccRCC by data standardization.

2.2 Clinical Validation for BATF in ccRCC

75 ccRCC tissue and 28 adjacent normal matched renal tissues were collected from the pathology department in EZhou Central Hospital. All these tissues were made tissue microarray and used for immunohistochemistry analysis.

2.3 Cell lines and culture.

The human ccRCC cell line 786-O and Caki-1 were purchased from the BeNa (ATCC CRL-1932) and the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences(TCHu135)respectively. The 786-O cell line was cultured in DMEM medium(Gibco;C11995500BT), while the Caki-1 cell line was cultured in

McCoy’s 5A medium (BasalMedia;L630). 10% fetal bovine serum (Bio-one Biotechnology;F05-001-B160216), penicillin(100 IU/ml) and streptomycin (100 μg/ml) were added into above the two mediums. And then the two cell lines were cultured at 37℃ in a humidified incubator containing 5% CO_2 .

2.4 Construction of vector plasmid

In this study, pHAGE-3x flag was selected as the vector, and its gene ID was nm_ 006399.3. The primers of target gene were synthesized by website software(https:// www. ncbi.nlm. nih. gov/tools/primer-blast/). The primers for synthesizing the target gene were h-BATF-F,ACGCATTCCACCAACCTCAT, h-BATF-R, ACAGTTCCCGTGTTCACTCC, respectively. The objective gene fragment was amplified by qPCR and the vector was digested and purified. The target gene fragment was connected to the above vector and an overexpression vector with flag marker was constructed. Then the recombinant vector was transferred into E. coli, and the positive clones were screened for amplification and culture.

2.5 Lentivirus packaging and target cell infection

In this experiment, 293T cell line was selected as packaging cell. pMD2-G (addgene; plasmid #12259 pmd2-g) and Pspax2 (addgene; asmid #12260 pspax2) were selected as packaging plasmids; the expression vector plasmids include control vector plasmid flag (m185-3ll MBL) and target gene vector plasmid BATF. In order to produce high titer virus particles, it is necessary to co transfect cells with expression vector and packaging plasmid at the same time. PEI was selected as transfection reagent (sigma; # gf95977287) and mixed with the above vector and plasmid for 15 minutes. After 48 hours, the virus was collected and centrifuged at 12000rpm for three minutes, and then centrifuged and collected the supernatant through 0.45μm-hole filter. The collected supernatant was directly added to 786-O cells and Caki-1 cells culture dishes for target cell infection.

2.6 CCK8 assay

The cell viability was detected by CCK-8 kit. Firstly, the two cell line suspensions were inoculated into 96 well plates, and 786-O cells and Caki-1 cells were inoculated into 100 μ L respectively. It was about 3 ~ 10 × 10^3 cells per well. Place the inoculated orifice plate in the incubator for 2-3h. The incubator shall be kept at a constant temperature of 37 ℃ with 5% CO_2 and humidity saturation. After the cells in the incubator adhered to the wall, replace the medium with the medium containing 10% CCK-8 reagent (Dojindo;44786), and add 120μ L to each well;786-O cells were cultured for 0h, 12h, 24h, 36h, 48h and 60H, and Caki-1 cells were cultured for 0h, 12h, 24h, 36h, 48h, 60H and 120h. The absorbance values of the two cell lines at 450 nm at each time cut-off point were detected by microplate reader.

2.7 EdU Assay

Taken 3000 cells of 786-O and Caki-1 in logarithmic growth stage to inoculate into each well of 96-well plate (thermo; 167008) and then be cultured to the normal growth stage at 37℃ and 5% CO2. EDU Kit (Roche;11647229001) was used to perform the DNA staining 24 and 48 hours later. After dyeing, the images were obtained under the microscope and the number of positive staining cells reflected the viability of cell proliferation .

2.8 The transwell migration assay

The stage of logarithmic growth of 786-O cells and Caki-1 cells were selected to be resuspended with DMEM or McCoy's 5A medium. Transwell upper chamber (Corning; 3421) was inoculated with 3×10^4 cells or 5×10^4 cells, meanwhile 600μL medium containing 2% serum or 10% serum was added to the Transwell lower chamber respectively. 786-O cells were cultured for 2h and 3h, Caki-1 cells were cultured for 10h and 24h, and washed twice with PBS. Then 600 μ L solution containing 4% paraformaldehyde was used to fix the cells at room temperature for 15 min. Finally, the cells were stained at 37℃ with 600μL 0.1% crystal violet (characters; 548-62-9) for 2h. Images were obtained by microscope. The more positive cells, the stronger the migration ability of cells.

2.9 Western blot

Polyacrylamide gel electrophoresis was used to extract and isolate proteins from the two cell lines and then the protein samples were transferred to a solid-phase carrier (nitrocellulose membrane). 5% nonfatdry milk was used to block the member in TBS-T buffer. And then the member was incubated with the primary antibody at 4℃ all night. With TBS-T buffer to rinse the blot and be incubated with the secondary antibodies for 1h. The westernblot bands were scanned and photographed. The molecular weight and net optical density of the target bands were analyzed by the gel image processing system.

2.10 Statistical analysis

Using one-way ANOVA to analyze the statistical difference among more than 2 groups. The variance homogeneity and demonstrating heteroscedasticity of data were analyzed by Bonferroni analysis and Tamhane's T2 analysis respectively. Using a two-tailed Student's t-test to analyze the differences between two groups. It was significant when $p < 0.05$.

3. Results

3.1 BATF expression in ccRCC from TCGA

In order to reveal whether BATF has a connection with ccRCC tumorigenesis, we got the clinical sample information through GEO and the largest sample size database from TCGA-KIRC（Kidney Renal Clear Cell Carcinoma）. The database (GSE66271, GSE76207) of GEO was analyzed and revealed that as compared to normal tissue, BATF expression was higher in tumor tissue (Fig. 1A).

In addition, subgroup analysis showed that the expression of BATF was significantly correlated with tumor stage and histopathological grade. A regular change was found that along with the increased the stage and grade of ccRCC, the expression of BATF was gradually up-regulated. (Fig. 1B and 1C). These results revealed that BATF was highly expressed in tumor tissue of ccRCC and maybe played an important role in the progression of ccRCC.

3.2 Clinical Validation in Chinese ccRCC patients

The clinicopathological parameters of ccRCC cases and the expression intensity of BATF were analyzed. It was found that BATF was highly expressed in ccRCC tissues (P = 0.0183). The expression of BATF was correlated with T stage (P = 0.0021), Fuhrman grade (P = 0.0000), tumor necrosis (P = 0.0000) and long-term prognosis (P = 0.0007), but not with tumor vascular invasion (P =0.8213) and patient age (P =0.5467). (Tab. 1) The IHC results of BATF low and over expression were showed as Figure 2.

3.3 BATF over expression inhibits ccRCC cell proliferation

In order to further study BATF in ccRCC , two cell lines of ccRCC with BATF overexpression, 786-O and Caki-1, were constructed to explore the role in migration and metastasis. Use westernblot to confirmed the successful establishment of BATF overexpression cell lines (Fig. 3A). Use CCK8 assay to evaluate cell viability and our results revealed that as compared to control group, the viability of the two cell lines with BATF overexpression were both significantly inhibited (Fig. 3B). We used EdU staining to detect the effect of BATF overexpression on ccRCC and as Fig. 3C showed that in the two cell lines with BATF overexpression the positive rate of EdU staining was significantly lower than that in the control group and this demonstrated that BATF overexpression may inhibit tumor progression by inhibiting cell cycle. These data suggested that BATF may act as a tumor suppressor gene to inhibit the progression of ccRCC.

3.4 BATF over expression inhibits ccRCC cell migration

Transwell migration experiment was used to investigate whether BATF affects the migration and invasion of cancer cells. As Fig.4A showed that the number of migrating cells in the two cell lines with BATF overexpression was significantly higher than control groups and proved by the statistics in Fig.4B. These suggested that BATF played an important role in tumor migration and metastasis. Targeting BATF may bring new strategies for the treatment of ccRCC.

3.5 BATF inhibits cell proliferation and migration via PI3K/AKT/mTOR pathway in ccRCC

In our study, we also found that the differential expression of BATF in ccRCC may be closely related to the signal pathway of PI3K/AKT/mTOR, so using westernblot to detect the activation of this signaling pathway. The westernblot results revealed that BATF overexpression decreased the level of phosphorylated PI3K ,AKT and mTOR compared with control cells in the two cell lines (Fig. 4A and B). Meanwhile, the BATF knockdown increased the level of phosphorylated AKT , PI3K and mTOR (Fig. 4C and D). And these suggested that BATF may regulate cell proliferation and migration in ccRCC through PI3K/AKT/mTOR signaling pathway.

Table 1
Distribution of BATF status in ccRCC according to clinicopathologic characteristics

Characteristics	Number	BATF expression		χ^2	*P*-value
		High	Negative/low		
ccRCC tissue	75	41	34	5.567	0.0183
Matched normal	28	8	20		
Age(years)					
60	62	28	34	0.3632	0.5467
≥60	41	21	20		
T stage					
I+II	89	37	52	9.4504	0.0021
III+IV	14	12	2		
Fuhrman grade					
I-II	78	27	51	21.633	0.0000
III-IV	25	22	3		
Tumor necrosis					
yes	26	23	3	23.314	0.0000
NO	77	26	51		
Vascular invasion					
yes	8	3	5	0.051	0.8213
NO	95	46	49		
Status					
Absent	23	18	5	11.392	0.0007
Present	80	31	49		

4. Discussion

BATF is a nuclear basic leucine zipper protein that mediates dimerization with members of the Jun family of proteins. BATF belongs to the AP-1/ATF superfamily and taken as a negative regulator in the events of AP-1/ATF transcription[7]. According to Gene Ontology annotations, BATF referred to DNA-binding transcription factor activity and sequence-specific DNA binding[8]. As we know, AP-1 family transcription

factor plays an important role in the differentiation of lineage-specific cells in the immune system. Such as, BATF is found to interact with JUNB to form heterodimer and then forms a complex with IRF4 in immune cells. After binding the AICE sequence (5'-TGAnTCA/GAAA-3'), an specific regulatory factor formed and mediated cooperative gene activation[9–10]. The mechanisms of BATF regulation are diverse. BATF also mediates T cell functions by regulating the pathway of PD-1/PD-Ls negatively. BATF was considered as one of specific transcription factors in tissue-resident memory T cells and was identified as the key target of REGNASE-1 and as a rheostat that shapes antitumour responses. The previous study showed BATF was proved that improving therapeutic efficacy by increasing the accumulation of REGNASE-1-deficient CD8+ T cells in the mouse models of melanoma and leukaemia. [11–12]. But the conclusions were inconsistent. There was also a research showed that BATF acts as an oncogene in non-small cell lung cancer and increases the proliferation of A549 cells and inhibits apoptosis[13–14]. Until now, the function of BATF in ccRCC has not been reported.

Previously, a consistent conclusion was reached by using bioinformatics technology and clinical data: with the increase of ccRCC stage and histopathological grade, the expression of BATF increased significantly. Therefore, there is a question: is BATF a tumor suppressor gene or an oncogene? So we constructed BATF overexpression cell lines (786-O-OE and Caki-1-OE) and verified it in vitro by traditional CCK8 and EdU staining methods. CCK8 assay and EdU staining were used to evaluate cell viability and proliferation of ccRCC respectively. According to these conventional assays, these results revealed that BATF overexpression inhibited ccRCC cell viability (Fig. 3B) and proliferation (Fig. 3C). And then, using Transwell chamber experiments to detect the migration potential of ccRCC cell, our result demonstrated that BATF overexpression inhibited migration of 786-O and Caki-1 renal cancer cells (Fig. 4A and 4B). These findings also suggest that BATF may be a tumor suppressor gene to inhibit the progression of ccRCC cell. Similar results were reported by Hao Huang[15], who demonstrated that BATF was identified as a better prognostic model from 143 methylation-driven genes in Colorectal Carcinoma. While the previous an inconsistent study showed that BATF promoted breast cancer cell migration and invasiveness by increasing TGFβ1 mRNA and protein levels[16]. Collectively these studies failed to reach a consistent conclusion and in ccRCC the prognostic inconsistency of BATF may be due to the heterogeneity of ccRCC specimens.

ccRCC is the most common kidney cancer, but its mechanism still needs to be clarified. As we know, the signaling pathway of PI3K/AKT has been proved dysregulated in many human diseases, especially cancer[17]. A study revealed that aberrant PC1 would promote progression of RCC through the increasing angiogenesis and activating PI3K/AKT/mTOR signal pathway[18], while another study revealed that GNE-477 would inhibited the cell growth of RCC in vitro and in vivo by reducing the activation of PI3K/AKT/mTOR cascade[19]. The previous studies showed that PTEN may be a favorable factor for ccRCC prognostic by regulating the signaling pathway of PI3K/AKT/mTOR[20] and CEP55 might be an effective prognostic marker in RCC by promoting EMT through PI3K/AKT/mTOR pathway[21]. Collectively, these previous studies indicated that the signaling pathway played an important regulator role in survival and proliferation of RCC cell. To the best of our knowledge, our study was the first report studying the interaction between BATF and the signaling pathway of PI3K/AKT/mTOR. The study found

that BATF overexpression decreased the level of phosphorylated AKT, PI3K and mTOR compared with control cells in the two cell lines (Fig. 4A and B). By contrast, the westernblot results also showed that BATF knockdown increased the level of phosphorylated AKT, PI3K and mTOR (Fig. 4C and D). Why is BATF underexpressed in paracancerous tissues and PI3K /AKT/mTOR signaling pathway still underphosphorylated? With the analysis of the differential expression of BATF in several clinicopathological features, the results demonstrated that the majority of the stratified prognosis was inconsistent with the overall prognosis. It can only be that other factors affecting PI3K /AKT/mTOR signal pathway in the microenvironment of ccRCC, which deserves our further investigation.

Conclusively, our findings demonstrated that BATF was overexpressed in ccRCC and could significantly inhibit cell proliferation and migration via the PI3K/AKT/mTOR signaling pathway and indicated that BATF may be an effective intervention target in ccRCC.

Abbreviations

BATF
Basic Leucine Zipper ATF-Like Transcription Factor
ccRCC：Clear-cell renal cell carcinoma
TCGA-KIRC
The Cancer Genome Atlas-Kidney Renal Clear Cell Carcinoma
VEGF
vascular endothelial growth factor
GEO
Gene Expression Omnibus

Declarations

Ethics approval and consent to participate

The Ethnics committee of the EZhou Central hospital approved the study and the written consent from all patients was obtained in the form of an institutional patient consent.

Consent for publication

Not Applicable

Availability of data and material

The datas used and material during the current study are available from the corresponding author on reasonable request.

Competing interests

No interest conflicts.

Funding

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Authors' contributions

ML performed the data analyses and wrote the manuscript FX Z reviewed the subject and be responsible for the correction of the paper. J J helped perform the analysis with constructive discussions; YZ C and Hong Zhang collected clinical samples of ccRCC. ZH Y and XL C collected and analyzed biological information data and conducted laboratory cell biological experiments. ZW L contributed to the conception of the study.

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Figures

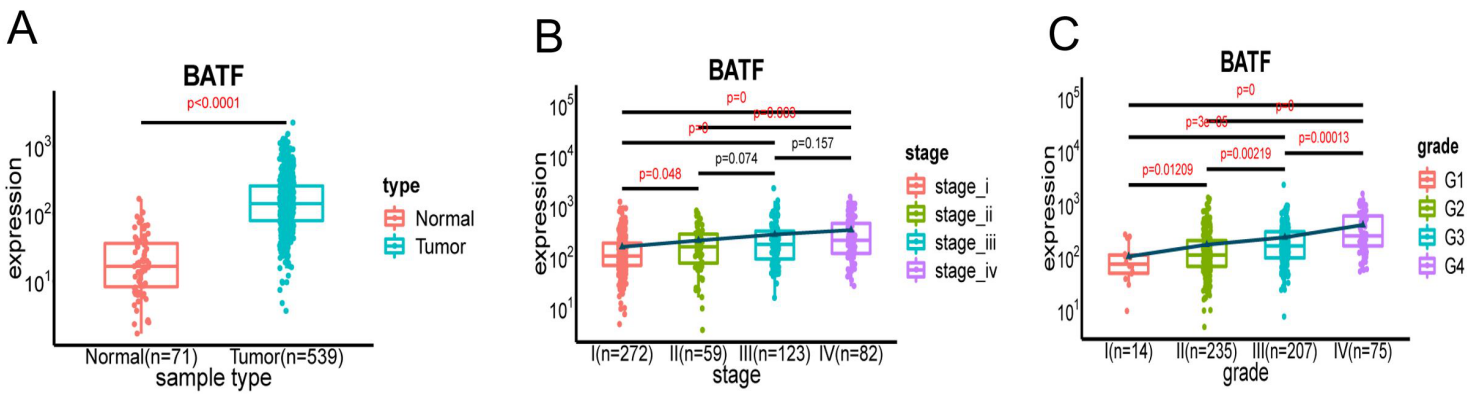

Figure 1

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Figure 2

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Figure 4

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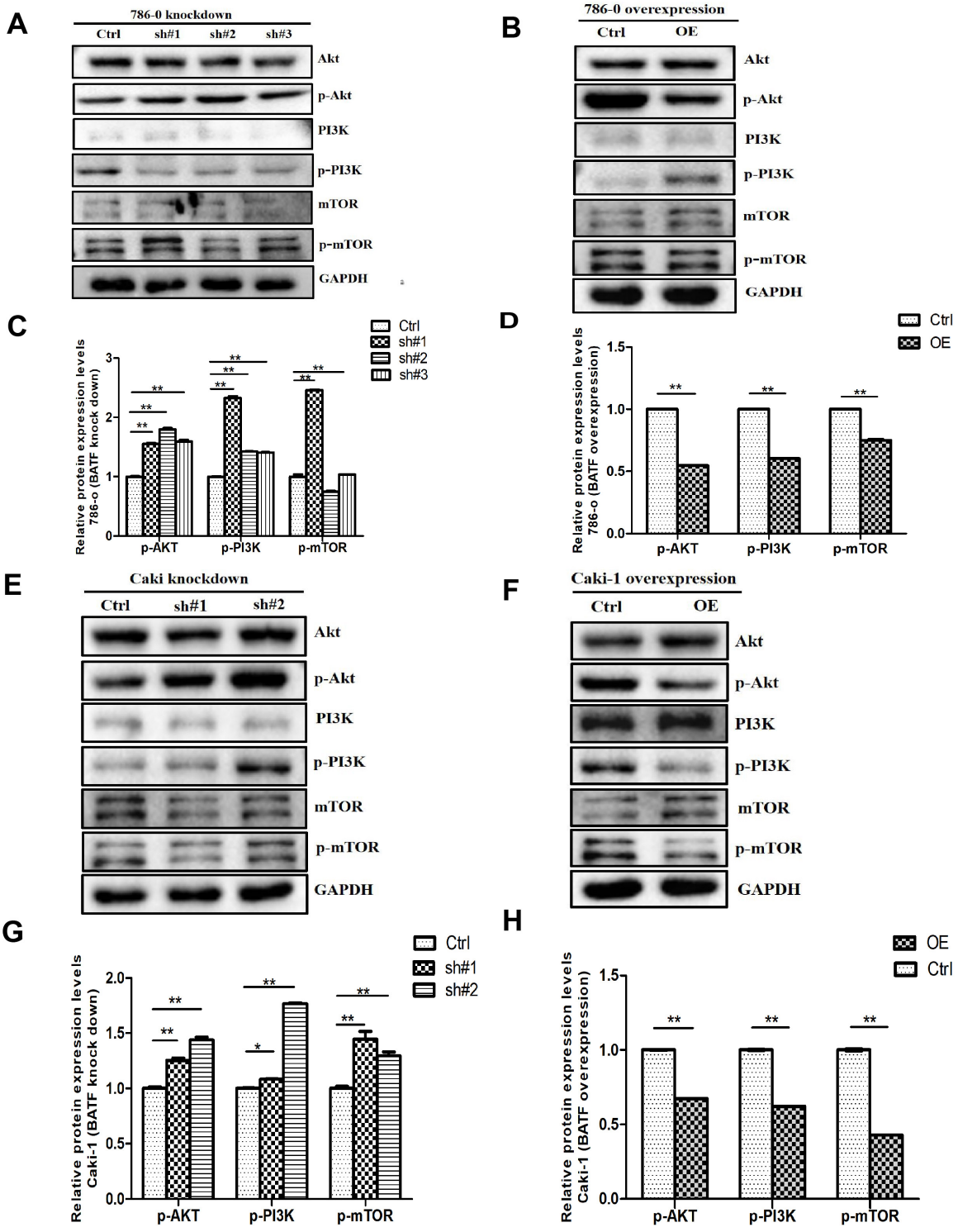

Figure 5

Caption not included with this version.