Gaα1 Promoted Proliferation, Migration and Invasion via Activating the Akt-mTOR/Erk-MAPK Signaling Pathway in Renal Cell Carcinoma

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Background: Renal cell carcinoma (RCC) accounts for about 2–3% of all adult malignancies. G protein alpha inhibitory subunit 1 (Gaα1) plays a key role in mediating PI3K-Akt signaling upon activation of receptor tyrosine kinases (RTKs). However, little is known about its expression, regulation and biological function in RCC.

Methods: Gaα1 expression in RCC tissues and cells was detected by quantitative real-time PCR (qRT-PCR), Western blot and immunohistochemistry (IHC). The effect of Gaα1 silence on cell proliferation and apoptosis of 786-O and ACHN cells was detected by CCK-8 assay and flow cytometry. Wound-healing assay and Transwell assays were used to detect the cell invasion in RCC cells. The expression of CDK4, cyclin D1, MMP-2, MMP-9, Bax, Bcl-2, p/t-Akt, p/t-Bcl-2 and p/t-Erk was detected by Western blot and qRT-PCR. Furthermore, a nude mouse subcutaneous xenograft model was used to further evaluate the potential effects of Gaα1 in vivo.

Results: In the present study, our data showed that Gaα1 expression was dramatically increased in RCC tissues compared with normal renal tissues. In addition, knocking down the expression of Gaα1 subsequently inhibited proliferation, migration and invasion of RCC cells in vivo and vitro. Furthermore, the expression of CDK4, cyclin D1, MMP-2 and MMP-9 was significantly reduced upon Gaα1 inhibition. Gaα1 positively regulates the activation of the mTOR and Erk pathways.

Conclusion: In conclusion, this study reveals Gaα1 promoted proliferation via activating the Akt-mTOR and Erk-MAPK signaling pathways in RCC, and Gaα1 may be a therapeutic and prognostic target for RCC.

Keywords: renal cell carcinoma, Gaα1, cell proliferation, cell migration, therapeutic targets

Introduction

Renal cell carcinoma (RCC) was the most common type of malignant tumor in kidney that accounts for more than 90% of renal cancer,1,2 among which 75–85% of them are clear cell renal cell carcinoma.3 RCC ranks among the 10 most common cancer types globally that causes about 140,000 deaths every year.4 Despite the continuous progress in medical treatment, the incidence of the disease has increased year by year.5 Patients with localized renal tumors generally have a favorable prognosis, with 5-year OS rates consistently between 60% and 70% after surgery.6 However, due to RCC is not sensitive to chemotherapy, the treatment options are very limited.7 The standard treatment for RCC is surgical resection.8

The prognosis of these patients with advanced disease is extremely poor, and the
5-year overall survival (OS) rate for patients with metastatic disease is less than 10%.8,9 Although immunotherapy, including interleukin-2 and interferon-α, is widely used in the treatment of metastatic renal cell carcinoma (mRCC), its efficacy is still controversial.10–12 Targeted drugs, including vascular endothelial growth factor receptor (VEGFR) inhibitors and rapamycin (mTOR) pathway inhibitors, provide new treatment regimens for mRCC patients. But the effect of these treatments is very limited and some patients suffer serious side effects such as hand–foot skin reactions, hypertension, diarrhea, etc.13,14 Thus, it is essential to identify the molecular mechanism underlying RCC to provide guidance for clinical decision-making and developing novel therapeutic strategies.

The G protein α inhibitory subunit (including Gαi1, Gαi2, and Gαi3) were initially identified by their ability to bind to GPCRs (G protein-coupled receptors) and inhibit adenylate cyclase (AC) activity.15 The traditional view is that G protein α inhibitory subunit (Gαi) routinely transduces G protein-coupled receptor (GPCR) signals, inhibits adenylate cyclase (AC) activity, and reduces the level of intracellular cAMP.16,17 Recent studies have shown that Gαi1/3 are physically bound to ligands that activated RTK (such as EGFR and KGF), which encodes and activates the adaptor protein Gab1 to mediate downstream Akt-mTOR activation.18–20 Upon stimulation by EGF, Gab1 can bind epidermal growth factor receptor (EGFR) as an adaptor protein, and promote proliferation and migration through the PI3K-Akt-mTOR pathway in vitro.21,22 In addition, Gαi1 mediates the activation of Akt-mTOR pathway induced by keratinocyte growth factor (KGF) and basic fibroblast growth factor (bFGF), and thus participates in the regulation of proliferation, differentiation, survival and migration of skin keratinocytes.19,20,22 In human gliomas, overexpression of Gαi1 leads to Akt activation and proliferation of glioma cells.23 However, the role of Gαi in the progression of RCC remains unclear.

This study aimed to investigate the expression and regulatory mechanisms of Gαi1 in RCC. Our study indicates that Gαi1 is highly expressed in RCC tissues. Our results further showed that suppression of Gαi1 expression significantly inhibited proliferation of RCC cells in vitro and the tumor growth of RCC cells in nude mice. In addition, the decline of CDK4, cyclin D1, MMP-2 and MMP-9 was significant after inhibiting Gαi1 in RCC cells. Furthermore, we found that the Akt-mTOR and Erk-MAPK pathways are downstream pathways of Gαi1. Together, our data suggest that Gαi1 may be a potential therapeutic target for RCC patients.

Materials and Methods

Clinical Samples

From 2017 to 2019, altogether 43 paired RCC and adjacent normal tissue samples were collected from patients who were undergoing renal cancer surgery in The Second Affiliated Hospital of Nantong University. The patients did not receive any treatment before surgery. All patients participating in the study were informed and written informed consent was obtained. The study was approved by The Second Affiliated Hospital of Nantong University Ethics Committee, according to the Declaration of Helsinki.

Cell Culture

Human renal cell carcinoma cell lines 786-O, A498, ACHN, Caki1 and Caki2 were purchased from the Shanghai Institutes of Biological Sciences (Shanghai, China). 786-O, Caki1 and Caki2 Cells were cultured in 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and A498, ACHN cells were maintained in MEM (Gibco) medium with 10% FBS in a humidified incubator (Thermo Fisher Scientific) air atmosphere containing 5% CO2.

Lentiviral Infection

Gαi1 expression was knocked down using lentiviral vectors. The LV-shGαi1 sequences were as follows: 5′-AGGATCAAAACACATGAA-3′ and LV-shNC sequences were as follows: 5′-TTCTCCCGAAGTGCAG-3′. Gαi1 knockdown (named as LV-shGαi1) lentivirus and negative control GV248 vector (termed as LV-shNC) infected the 786-O and ACHN cells, following the reagent manufacturer’s instructions (Genechem, Shanghai, China). Green fluorescence cells were observed with the fluorescence microscope and retroviral production and puromycin stable cell selection has been previously described cell infection efficiency was evaluated. The knockdown efficiency was determined by qRT-PCR and Western blot.

Western Blotting Analysis

Proteins were extracted from RCC patient tissues or cells in ice-cold RIPA buffer containing protease inhibitors and protein concentration was determined by BCA Protein Assay Kit. Western Blot assays were performed by well-established protocols as previously described.27 Image J was used for
density analysis to quantify the intensity of the Western blotting band. The primary antibodies used in Western blotting were as follows: Gai1 (1:1000, Proteintech), β-actin (1:5000, Proteintech), Akt (1:1000, CST), p-Akt (1:1000, CST), Erk (1:1000, CST), p-Erk (1:1000, CST), S6 (1:1000, CST) p-S6 (1:1000, CST), cyclin D1 (1:2000, Proteintech), CDK4 (1:1000, Proteintech), MMP-2 (1:1000, Proteintech), MMP-9 (1:1000, Proteintech), BAX (1:4000, Proteintech), Bcl2 (1:1000, Proteintech). The secondary antibody used in Western blotting was HRP goat anti-rabbit IgG (1:5000, Proteintech) and HRP goat antimouse IgG (1:5000, Proteintech).

RNA Isolation and Quantitative Real-Time PCR
Total RNA was isolated from RCC patient tissues or cells lysates using Trizol kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized using a Thermo-script RT kit (Life Technologies, Rockville, MD, USA). Quantitative real-time PCRs were performed in in CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA), using SYBR PCR reagent (Takara, Shiga, Japan). GADPH is standardized as a gene expression level. The sequences of primers are as follows:

Gai1-F: GCTCAACCAAAATTACATCCGGAC
Gai1-R: ATCTCTGACCTCCACATCAAC
CDK4-F: TGGTGATGTCATTCACACAGA
CDK4-R: TTGATGAGGGGAAAGGAAATGC
Cyclin D1-F: TTCCTGACCTTAAAGATGAGG
Cyclin D1-R: GTTCCAACCTTGAAGCTTGCACC
MMP-2-F: AGACCTGGTGCAAGAAAGAAC
MMP-2-R: TCTGTGACCTGCAAGGTC
MMP-9-F: TCGAGAACCTTGCAGGGCGCTGT
MMP-9-R: ATGGACCGTGCTTGTACGTATG
BAX-F: CAGCTGACATGTTCCTGACGG
BAX-R: AGGCGATGATGTTTCGTACAG
Bcl2-F: ATGTGGCCTTCTTGAAGTC
Bcl2-R: TCTGGTACATGTCATCCACAG
GAPDH-F: ACTTGGTATCGTGAAAGGACTC
GAPDH-R: GTAGAGGCCAGGATGTCTTCTG

Immunohistochemistry
Renal cancer and normal tissue were used for immunohistochemistry. After baking for 60 min in a constant temperature oven at 60°C, tissue chips were dewaxed in xylene and hydrated in graded alcohols. Slides were blocked for 5 min, then primary antibody Gai1 (1:100, Proteintech), Ki67 (1:8000, Proteintech) was added for incubating at 4°C overnight. After washing, secondary antibody HRP Goat Anti-Rabbit IgG (1:200, Proteintech) was added and incubated for 2 h at room temperature. Finally, the tissue specimens were stained with diamino-benzidine and examined with microscopic. Specimens were classified into negative, positive, ++ positive, or +++ positive, based on the sum of the staining intensity (varied from weak to strong) and staining extent scores.

CCK-8 Assay
Cell proliferation was measured by CCK-8 assay (CCK-8, Dojindo, Japan). Lentivirus infected 786-O and ACHN cells in exponential growth phase were seeded onto five 96-well plates (3×10^5 cells/well) in triplicate and cultured for 1, 2, 3, and 4 days. Four hours before absorbance measuring, 10 µL of CCK-8 solution was added. The absorbance was measured at 450 nm with a microplate reader after incubated at 37°C for 2 h.

Colony Formation Assay
Lentivirus infected cells 786-O and ACHN were seeded into 6-well plates (800 cells/well) and cultured in the RPMI-1640 or MEM medium with 20% FBS for 7 days. The colonies were fixed with 4% paraformaldehyde for 30 min. The colonies were then washed with PBS and stained with 0.1% crystal violet. Finally, The cell colonies were photographed.

Wound Healing Assay
786-O and ACHN cells (5×10^5 cells/well) were seeded on a 6-well plate. The cell layer scratches in each well were incubated with serum-free medium. Take pictures with the microscope every 6 hours, and repeat 3 times to calculate the cell migration rate of each group.

Transwell Assay
Transwell kit (8.0μm pore size polycarbonate filter) with a Matrigel overlay (BD, NJ) was used to evaluate the invasion ability of cells. 786-O cells and ACHN cells (1×10^5) with 200μL of FBS-free medium was added into the upper chamber, and 600μL of medium with 20% FBS was add into the lower chamber. After incubating for 24 hours at 37°C with 5% CO2, non-invasive cells were removed on the upper surface by a cotton swab. The invaded cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted.
Cell Apoptosis Were Detected by Flow Cytometry

To further quantify apoptotic cells, flow cytometry (FCM) analysis was performed according to the manufacturer’s instructions. 786-O and ACHN cells were seeded in a 6-well plate and cultured for 24 hours. After centrifugation (1000×g), the cells returned to normal and were suspended in binding buffer, and stained with 5μL PE Annexin V and 5μL 7-AAD. At least 5×10^3 cells/sample were collected and analyzed using an EPICS XL flow cytometer (BD LSR Fortessa, USA).

In vivo Xenograft Experiments

Female BALB/c nude mice aged 4–6 weeks were obtained from the Animal Research Center of
Nantong University. Approximately 5.0×10^6 different 786-O cells (786-O-shNC, and 786-O-shGαi1) were subcutaneously injected into the left and right sides of the nude mice armpit. The mice body weight and tumor volume were measured every other week. The tumor volume calculation formula: tumor volume (mm^3) = length×width^2×0.52. All mice were euthanized at t30 days after seeding 786-O cells and tumors removed, weighed, fixed and embedded in IHC. The protocol for animal experiment was approved by the Animal Ethics Committee of Nantong University and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Statistical Analysis**

Statistical analysis between the two groups was analyzed by Student’s t-test and comparisons involving multiple groups were analyzed by two-way ANOVA using GraphPad 5.02, followed by post hoc tests. All data are expressed as the mean ± SD, and p < 0.05 was considered represent statistically significant. All experiments were repeated at least three times.

**Results**

**Gαi1 Expression is Upregulated in RCC Tissues and RCC Cell Lines**

To investigate the role of Gαi1 in RCC, the mRNA and protein expression levels were detected by qRT-PCR,
Western blot and immunohistochemistry (IHC) in a series of RCC cell lines and 43 pairs of RCC tissue samples (non-metastatic tumors and their adjacent normal tissue specimens). qRT-PCR result suggested that Gαi1 mRNA expression was significantly upregulated in fresh RCC tissues (Figure 1A). Western blotting and IHC analysis showed a significant increase of Gαi1 protein expression in RCC (Figure 1B and E). The expression of Gαi1 in five RCC cell lines examined by qRT-PCR and Western blotting revealed that Gαi1 is highly expressed in RCC cell lines (Figure 1C and D).

Gαi1 Knockdown Inhibited RCC Cells Proliferation and Migration in vitro

In order to further investigate the underlying mechanism of Gαi1 in promoting RCC, we knockdown Gαi1 expression in
RCC cell line 786-O cells and ACHN cells by lentiviruses-mediated expression of control shRNA (LV-shNC) and Ga11-specific shRNA (LV-shGa11) in vitro. The mRNA and protein expression of Ga11 in 786-O and ACHN cells after virus infection was detected by Western blotting and qRT-PCR, which demonstrates efficient downregulation following infection with LV-shGa11 (Figure 2A–D).

To understand the role of Ga11 in the proliferation of RCC cells, we first performed a CCK-8 assay and a colony formation assay. CCK-8 assay demonstrated that the reduced proliferation of 786-O and ACHN cells after Ga11 knocking down when compared to control cells (Figure 3A). Also, results from the colony formation assay indicated that silencing Ga11 obviously inhibited the cell proliferation, which was demonstrated by the number of 786-O and ACHN cell colonies is significantly reduced (Figure 3B and E). Then, the cell migration of 786-O and ACHN cells was further evaluated (Figure 3C and F). The wound healing assay and transwell assay indicated that Ga11 knockdown significantly impaired the invasive and migratory capabilities of 786-O and ACHN cells (Figure 3D and G). Furthermore, significant decline of CDK4, cyclin D1, MMP-2 and MMP-9 was observed after inhibiting the function of Ga11 in 786-O and ACHN cells (Figure 4A–E).

**Ga11 Knockdown Promoted Cells Apoptosis in vitro**

Apoptotic dysfunction caused by dysregulation of apoptosis-related proteins plays an important role in the development of cancer. To determine whether Ga11 influenced RCC cell apoptosis, flow cytometry assay

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**Figure 4** Ga11 knockdown inhibited the expression of CDK4, cyclin D1, MMP-2 and MMP-9 in RCC cells. (A) Western blot analysis of CDK4, cyclin D1, MMP-2 and MMP-9 protein expression in 786-O and ACHN cells with Ga11 knockdown. (B–E) The mRNA expression of CDK4, cyclin D1, MMP-2 and MMP-9 was detected by qRT-PCR in 786-O cells of shNC and shGa11 groups. Results were presented as mean ± SD. *P<0.05, **P<0.01.
was performed to detect apoptotic cells. We observed that Gαi1 knockdown obviously promoted apoptosis of 786-O and ACHN cells, respectively (Figure 5A and C). Besides, the mRNA expression and protein expression of Bax and Bcl-2 were detected by qRT-PCR and Western blot. The result of qRT-PCR and Western blot analysis showed that the expression of Bax was up-regulated and Bcl-2 was down-regulated in the Gαi1 knockdowning RCC cells (Figure 5B–E). These results indicated that knockdown of Gαi1 induced apoptosis of RCC cells.

**Gαi1 Knockdown Inhibited mTOR and Erk Signaling Pathways**

Many lines of evidence show that Gαi1 forms a complex with RTKs (FGFR, EGFR and KGFR) and mediates the
activation of downstream Gab1-PI3K-Akt signaling pathway. To investigate the efficacy of Gai1 in the signaling pathways, we used Western blot analysis to examine the activation of these pathways. We found that knockdown of Gai1 inhibited the ratio of p-Akt/t-Akt, p-S6/t-S6 and p-Erk/t-Erk in both 786-O and ACHN cells (Figure 6A and B). These results indicate that knocking down Gai1 inhibits the Akt-mTOR and Erk-MAPK signaling pathways.

Gai1 Knockdown Inhibited Tumor Growth in Nude Mice

According to previous results that knockdown of Gai1 led to impaired proliferation of cancer cells, we further analyzed whether Gai1 shRNA has an effect on tumor growth in vivo. To establish mice xenograft models, 786-O cells with or without Gai1 knockdown were injected subcutaneously into nude mice respectively. Our results showed that tumor growth was significantly impaired in the group of Gai1 knockdown compared with the control group (Figure 7A and B). And knockdown of Gai1 resulted in a significant reduction in tumor weight as assessed at the end of experiment (Figure 7C and D). We found that Ki-67 staining was stronger in the control group compared to Gai1-knocking down group (Figure 7E). In summary, the above results indicated that Gai1 knockdown could inhibit RCC tumor growth in vivo.

Discussion

Renal cancer have no specific presenting symptoms or signs. It has been reported that approximately 30% of RCC patients have a metastatic lesion at the time of initial diagnosis. The clinical prognosis of patients with metastatic RCC (mRCC) is extremely poor, with a median survival of only 13 months. The Akt/mTOR signaling pathway plays a very important role in the occurrence and development of renal cell carcinoma; therefore, targeting mTOR and block the Akt/mTOR signaling pathway is an potential effective way to treat mRCC. mTOR inhibitors are mainly targeted at mTORC1, which suppress tumor cells growth upon treatment of a variety of cell growth factors including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). The related drugs such as temsirolimus and everolimus were approved by the US FDA for the treatment of progressive metastatic RCC. However, most RCC patients have a shorter period of effective response to mTORC1 inhibitors, and eventually most patients acquire resistance. Therefore, there is an urgent need to find new molecular mechanisms to treat RCC and improve the prognosis of patients with metastatic RCC.

The results of this study indicate that Gai1 may be a new anti-cancer target protein to slow down the growth of RCC cells. First, Gai1 mRNA and protein are up-regulated in human RCC tissue (compared to surrounding kidney tissue) and RCC cells (compared to human normal renal tubular epithelial cells). Second, Gai1 can promote RCC cell proliferation, migration and invasion, and inhibit RCC cell apoptosis. Third, the tumor growth of Gai1 knocking down RCC cells was slower than that of control tumors. Fourth, in RCC cells, Gai1 can activate the Akt-mTOR and Erk-MAPK signaling pathways.

Gai protein is essential for EGF to activate the PI3K/Akt/mTORC1 pathway, while EGF and EGFR are commonly
expressed in most human cancers.\textsuperscript{32} The activation of Akt and mTOR is important for tumor proliferation, survival, and metastasis. The function of Ga1 in the progress of the cancer is crucial,\textsuperscript{33} so Ga1 protein is likely to become a potential new target for anti-cancer therapy. The results of immunohistochemistry in this study showed that Ga1 was highly expressed in RCC tissues, and the up-regulation of Ga1 in RCC tissues was related to tumor staging, which suggests that Ga1 plays an important role in the occurrence and development of RCC. Similar studies have shown that Ga1 is highly expressed in human glioma tissue, and the up-regulation of Ga1 in glioma tissue is related to tumor grade and Akt activation. This study showed that Ga1 can promote the proliferation, migration and invasion of 786-O and ACHN cells in vitro. The establishment of subcutaneous xenogeneic tumor formation experiments in nude mice showed that Ga1 can promote the proliferation of RCC cells.

The Akt/mTOR signaling pathway plays a vital role in the occurrence and development of renal cancer,\textsuperscript{34} and studies have shown that Ga1 protein is essential for EGF to activate the PI3K/Akt/mTORC1 pathway.\textsuperscript{20} Therefore, this study also explored whether Ga1 protein can regulate the proliferation, migration and invasion of RCC cells by activating the mTOR and Erk signaling pathway. Western
Blot experiments showed that knockdown of Gai1 inhibited the ratio of p/t-Akt, p/t-S6 and p/t-Erk in 786-O and ACHN cells.

In conclusion, we found that Gai is highly expressed in human RCC tissues. And our further research shows that Gai protein can regulate the proliferation, migration and invasion of RCC cells by activating the Akt-mTOR and Erk-MAPK signaling pathways. Therefore, we conducted a preliminary discussion on the role of Gai protein in the development of RCC, which provides a new and potential therapeutic target for the treatment of RCC.

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**Disclosure**

The authors declare no conflict of interests.

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