Aberrant MEK5 Signalling Promotes Renal Cancer Development via mTOR Activation

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Research Article

Keywords: Clear cell renal cell carcinoma, MEK5, mTOR, inhibitor

Posted Date: November 30th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1074666/v1

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Abstract

**Purpose:** Renal cell carcinoma is one of the most incident malignancies globally. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma. However, comprehensive clinical treatment has some limitations. Therefore, exploring the pathogenesis and identifying novel therapeutic targets are required urgently. MEK5 has been reported to play an essential role in the development of various cancers. However, no study has evaluated its role and specific mechanisms in ccRCC.

**Methods:** Using the ONCOMINE database, MEK5 expression in ccRCC and normal tissues was compared. ccRCC and adjacent normal tissues were collected from fourteen ccRCC patients, and ccRCC expression was assessed by qPCR and immunohistochemistry. MEK5 overexpression and knockdown plasmids were constructed and transfected into ccRCC cells. CCK8, wound-healing assay, and clone formation assay were performed to examine the cell proliferation, migration, and clone formation ability of ccRCC cells. Furthermore, a western blot was performed to verify the regulation and influence of MEK5 on the mTOR signaling pathway. The MEK5 small molecule inhibitor BIX was used to treat cells, followed by CCK8, wound-healing assay, clone formation, and flow cytometry assay to examine the cell proliferation, migration, clone formation ability, apoptosis, and cell cycle. Finally, a murine subcutaneous tumor model was constructed, and the effect and safety of BIX were evaluated in-vivo.

**Results:** The ONCOMINE database indicated that the MEK5 expression in ccRCC was significantly higher than the normal tissues, further confirmed in clinical specimens. The knockdown of MEK5 markedly inhibited the ability of ccRCC cell proliferation, colony formation, and migration. In contrast, MEK5 overexpression promoted cell proliferation, colony formation, and migration. Western blotting showed that overexpression of MEK5 can further activate the mTOR signaling pathway. The MEK5 inhibitor, BIX treatment of ccRCC, significantly inhibited cell proliferation, arrested the cell cycle in the G0/G1 phase, induced apoptosis, and effectively inhibited cell migration and clone formation. BIX also showed an excellent anti-tumor effect and favorable safety profile in murine models.

**Conclusions:** MEK5 regulated the mTOR signal pathway and regulated the cell proliferation, cycle, migration, clone formation of ccRCC. Targeted inhibition of MEK5 had a good anti-tumor effect and favorable safety profile, providing new directions for ccRCC therapy.

Introduction

The incidence of renal cell carcinoma is steadily increasing annually. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma and accounts for most renal cancer deaths [1]. Localized renal cell carcinoma can be treated by partial or radical nephrectomy [2], ablation [3], and active monitoring [4]. However, approximately 30% of patients have metastases or relapses after partial or radical nephrectomy [5, 6].
Conventional cancer therapies, including radiation and chemotherapy, are ineffective in treating metastatic ccRCC [7]. Therefore, comprehensive treatment is crucial. High-dose interleukin 2 (IL2) is one of the first cytokines, which, along with interferon α (IFN-α), was used for the treatment of RCC, but only 15-25% responded to this treatment [8]. The treatment options for ccRCC currently include targeted therapy and checkpoint inhibitor (CPI) immunotherapy. The targeted therapies include vascular endothelial growth factor receptor, tyrosine kinase inhibitors (VEGFR, TKIs), and the mammalian target of the rapamycin (mTOR) pathway signaling inhibitors. Further, a majority of patients initially respond to therapy, most eventually relapse or progress [9]. Therefore, the dynamic development of new therapeutic drugs or the search for novel therapeutic targets is essential for the clinical treatment of ccRCC.

The mitogen-activated protein kinase (MAPK) signaling pathway controls diverse cellular processes in response to various extracellular stimuli. There are four well-known typical MAPK subfamilies: ERK1/2, c-Jun NH2-terminal kinases (JNK), p38 MAPK isoforms, and ERK5. Three atypical MAPK subfamilies: ERK3/4, ERK7, and NLK [10]. The mitogen extracellular signal-regulated kinase 5/extracellular signal-regulated kinase 5 (MEK5/ERK5) pathway is the lesser-studied segment in the MAPK family. The MEK5 encodes a 444-amino-acid protein which displays approximately 40% identity to other MEKs. ERK5 is activated in response to cell stress and mitosis through its selective phosphorylation by MEK5 [11]. The activation of MEK5/ERK5 regulates various cell activities, such as cell proliferation, differentiation, migration, and apoptosis [10].

MEK5/ERK5 was found to be associated with cancer due to its abnormal expression in human tumors, including colon cancer [12], breast cancer [13], prostate cancer [14], hepatocellular carcinoma [15]. The overexpression of MEK5/ERK5 in various tumors makes it an ideal target for targeted therapy. The role of MEK5 in ccRCC and the underlying mechanism is still unclear. This study systematically described the function and role of MEK5 and its downstream signaling pathways in ccRCC for the first time. Thus, confirming that MEK5 is specifically and highly expressed in tumor tissues, regulating tumor cell proliferation, cycle, invasion, and migration, and targeting MEK5 has a good anti-tumor effect in-vivo and in-vitro.

Materials And Methods

Plasmids, Cell Lines, and Reagents

Human RCC lines (A498, 769-P) were obtained from the American Type Culture Collection (Manassas, VA, USA), maintained in our laboratory, and cultured under standard conditions. Cells were cultured in RPMI-DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL).

The MEK5 inhibitor BIX was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Dimethyl sulfoxide (DMSO) was acquired from Sigma-Aldrich (St. Louis, MO, USA) and Cell Counting Kit-8 (CCK-8)
from MedChemExpress (Monmouth Junction, NJ, USA). Antibodies to MEK5, p-mTOR, p-4EBP1, p-p70, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

MEK5 knockdown and overexpression plasmid were purchased from the DNA library of Shanghai Jiao Tong University School of Medicine (Shanghai, China).

**Real-time fluorescence quantitative PCR (RT-qPCR)**

RT-qPCR was performed following the instructions of the TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China), and all the primers were synthesized by Sangon (Sangon Biotech Co., Ltd., Shanghai, China).

MEK5 forward: 5’-CCTTCCAGTTGGAGAGTTCTCG

MEK5 reverse: 5’-CGGCATTTCCATCATTGAACTGC

Actin forward: 5’-CACCATTGGCAATGAGCGGTTC

Actin reverse: 5’-AGGTCTTTGCGGATGTCCACGT

**Western blotting**

The whole-cell lysates were prepared by direct lysis with 1× SDS sample buffer and separated using 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA, USA). After blocking the membrane with 5% non-fat milk prepared in +0.1% Tris-buffered saline, the membranes were incubated with primary antibodies overnight at 4°C. This was followed by an hour incubation with horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology, Beverly, MA, USA). The protein bands were detected using a chemiluminescence phototope-HRP kit (Cell Signaling Technology).

**Detection of apoptosis**

The eBioscience™ Annexin V-APC apoptosis detection kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect apoptosis. Briefly, ccRCC cells were treated with BIX solution for the indicated times, ccRCC cells (1 × 10^6) were harvested, washed with PBS, and resuspended in the binding buffer. Annexin V-APC (5 µL) and PI (5 µL) were sequentially added to the cell suspension and incubated for 15 min in the dark at room temperature. Cells were analyzed using a flow cytometer (Fortessa, San Francisco, CA, USA) and DIVA™ v8.0 software. Approximately 10,000 cells were analyzed from each sample.

**Cell cycle assay**

Cell cycle distribution was analyzed by estimating the DNA content using flow cytometry. Harvested cells were fixed with 75% ethanol at −20°C for 24 h. The samples were then incubated with 50 μg/mL RNase A for 30 min at 37°C, followed by 100 μg/mL propidium iodide (PI) at 37°C for 30 min. The DNA content was analyzed by a flow cytometer (Fortessa), and cell cycle distribution was estimated using FlowJo software.
Wound-healing assay

Cells were grown to confluence in 100 mm$^2$ dishes, and artificial wounds were created using a sterile 200 µL pipette tip by scraping. The cells were allowed to close the wound and photographed at the indicated times.

Colony Formation Assay

The cells were subcultured until reaching the logarithmic growth phase, the stable transfected cells were digested with 0.25% trypsin and gently pipetted to make them single cells. The cells were counted and reseeded at a density of 1000–2000 cells per well into a 6-well plate and incubated at 37°C with 5% CO$_2$ in a humidified incubator for 10–15 days. When the cell formed visible clones, cells were terminated cultured, and washed twice with PBS, fixed using 4% paraformaldehyde for 15 min, and then stained using Crystal Violet for 5 min. The cells were then washed with PBS and dried.

Immunohistochemical analysis

Isolated tumors was formalin-fixed and paraffin-embedded and sectioned into slices. The tissue sections were subsequently stained with hematoxylin and eosin(HE) using standard techniques and used for the MEK5 and anti-Ki67 immunoassays.

Xenograft mouse model

The whole animal experimental protocol was approved by the Shanghai Jiao Tong University School of Medicine Institutional Animal Care and Use Committee(Shanghai, China). BALB/c nu/nu mice (female, aged 4 weeks) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and kept in specific pathogen-free(SPF) condition in the Animal Center at Renji Hospital,shanghai, China. A human ccRCC model was established by inoculating subcutaneously 1 × 10$^6$ A498 cells near the armpits of the forelimbs of experimental mice. The mice were randomly divided into the BIX and control groups when tumor masses were visible. BIX was intraperitoneally administrated at a dose of 30 mg/kg once a day for subsequent 10 days. The tumor growth was monitored by daily measurements by electronic cliper, in two dimensions(length(L) and width(W) ), and tumor volume(V) was estimated using with the formula: V=L/2 ×W2. After ten days treatments, the mice were sacrificed by cervical dislocation, and the tumor masses were removed, photographed and weighed.

Patient's Samples and Analysis

Under the supervision of the local ethics committee and pathologists, and without interfering with histological evaluation, fresh samples of four cases were obtained from ccRCC patients, diagnosed and treated at the Department of Urology, Renji Hospital. T test was used to evaluate the difference between MEK5 expression in ccRCC and adjacent normal tissues.

Statistical analysis
The statistically significant differences observed in drug-treated versus control cultures were determined using the Wilcoxon signed-rank test. The minimum level of significance was $p < 0.05$.

**Result**

**MEK5 is highly expressed in ccRCC tissues**

The MEK5 mRNA expression in ccRCC patient samples was determined using the ONCOMINE database. MEK5 mRNA expression in normal and tumor tissues was compared in the Oncomine database. MEK5 mRNA expression was significantly upregulated in tumor tissues compared to the normal kidney tissues (Fig. 1A). ccRCC samples were collected to detect MEK5 at mRNA levels. The mRNA levels of MEK5 expression in ccRCC tissues were significantly higher than in adjacent tissues ($p<0.05$) (Fig. 1B). Immunohistochemical staining data were available from fourteen normal renal and ccRCC tissues. MEK5 exhibited a higher expression in the ccRCC tissues than normal tissues ($p<0.05$)(Fig. 1C). Combined with the database and clinical specimen data, we found that MEK5 was specifically and highly expressed in ccRCC tumor tissues.

**MEK5 promoted proliferation, migration, and colony formation**

MEK5 was specifically and highly expressed in ccRCC tumor tissues. The biological function of MEK5 in ccRCC was validated further. Two MEK5 knockdown plasmids were generated, and the knockdown efficiency of MEK5 was measured at the protein and mRNA levels (Fig. 2A). MEK5 knockdown suppressed the proliferation of ccRCC cells compared with the control (Fig. 2B). Conversely, overexpression MEK5 significantly promoted cell proliferation, as shown in Fig. 2D. The cell cycle effect of MEK5 was evaluated(Fig. 2E and 2F), suggesting that knockdown of MEK5 can induced cell cycle arrested in G0/G1 phase. Cell migration is one of the important processes in tumor development and metastasis. The migration effects of MEK5 on the ccRCC cell lines were evaluated (Fig. 2G and 2H), suggesting that MEK5 can promote the migration of ccRCC cells. Additionally, the effect of MEK5 on colony formation of ccRCC cells was examined. Consistent with the results of the CCK-8 assay, colony formation analysis demonstrated that the MEK5 knockdown suppressed the colony formation of ccRCC cells, whereas MEK5 overexpression increased its colony formation capacity (Fig. 2I and 2J).

**MEK5 regulates the mTOR signaling pathway**

MEK5 plays an important oncogene effect in ccRCC, and its mechanism was explored further. It has been reported that MEK5 can regulate the mTOR signaling pathway, which plays a vital role in the development and progression of ccRCC. Thus, the effects of MEK5 on the mTOR signaling were examined. The knockdown of MEK5 inhibited the mTOR signaling pathway, while overexpression activated the mTOR signaling pathway (Fig. 2K and 2L).
Targeted inhibition of MEK5 has a good anti-tumor effect in-vitro

MEK5 activates the mTOR signaling pathway and promotes proliferation, migration, colony formation, and tumor growth. The effects of MEK5 inhibitors on ccRCC cells were evaluated. The MEK5 inhibitor BIX efficiently induced cell apoptosis and inhibited cell proliferation of ccRCC (Fig. 3A and 3B), consistent with the knockdown of MEK5. The G0/G1 arrest effect of BIX on ccRCC cells in the G0/G1 phase was dose-dependent (Fig. 3C). The front experiments confirmed that MEK5 knockdown suppressed colony formation and migration of ccRCC cells. The same phenomenon was observed in the experiment of BIX targeted inhibition of MEK5 (Fig. 3D and 3E). BIX effectively inhibited the activation of the mTOR signaling pathway in ccRCC cells (Fig. 3F). The results of the experiments showed that the MEK5 inhibitor BIX has a good anti-tumor effect in-vitro.

Targeted inhibition of MEK5 has a good anti-tumor effect in murine models

We further validated the experiment in-vivo to evaluate the anti-tumor effect and safety of BIX. The murine’s subcutaneous tumor models were constructed using A498 cell lines, and the tumor of murine in the DMSO group and the MEK5 inhibitor BIX group were observed dynamically. MEK5 inhibitor BIX effectively reduced tumor growth and load in murine (Fig. 4A and 4B). Besides, there was no significant difference in body weight (Fig. 4C), excretion, and life status between the two murine groups, suggesting a favorable safety profile of MEK5 inhibitor BIX in-vivo. The tumor tissues were processed for immunohistochemical analysis, and the results are shown in Fig. 4D. MEK5 inhibitor BIX effectively reduced tumor growth in murine by reducing Ki67 expression.

Discussion

MEK5/ERK5 pathway is involved in multiple processes, such as cell proliferation, differentiation, motility, and apoptosis. It has attracted attention because of its role in tumors [11, 16, 17]. High expression of MEK5 in metastasis prostate cancer may be correlated to bone metastasis, and the AP-1-induced by MEK5/ERK5 makes prostate cancer more aggressive and harbors poorer prognosis [14]. In small cell lung cancer (SCLC), the abnormal expression of MEK5/ERK5 plays a pivotal role in driving tumors through modulating lipid metabolism pathways [18]. In this study, we confirmed for the first time that the MEK5 was highly expressed in ccRCC and played a crucial role in cell proliferation, migration, and colony formation, and also demonstrated that MEK5 activated the mTOR signaling pathway in ccRCC.

The mTOR signaling pathway regulates cell growth, proliferation, motility, survival, and metabolism [19, 20]. Aberrant activation of the mTOR pathway has been reported in various tumors, including acute lymphoblastic leukemia (ALL), breast cancer, prostate cancer, head, and neck squamous cell carcinoma [21–25]. Two mTOR inhibitors (temsirolimus and everolimus) have been approved as medications for metastatic ccRCC [26]. The mTOR pathway has a central role in regulating the immune response [27, 28].
Tumor treatment and immunotherapy can be improved because of the effect of mTOR inhibitors on specific T cell subsets. In a phase I clinical trial of combined use of everolimus and low-dose cyclophosphamide in the treatment of metastatic RCC, it was observed that changes in various immune cells populations promoted anti-tumor immunity [29, 30]. In a mouse model of renal cell carcinoma, everolimus combined with anti-PD-L1 was more effective in reducing tumor burden than PD-L1 treatment alone [31]. Our research confirmed that MEK5 is involved in the abnormal expression and activation of mTOR and the occurrence and development of ccRCC.

MEK5 inhibitors have a good application prospect in various tumors by targeting the biological activity of MEK5. MEK5/ERK5 inhibitors combined with PI3K/Akt inhibitors were more effective than either inhibitor alone in reducing the proliferation and survival of triple-negative breast cancer (TNBC) [32]. MEK5 inhibitor may cause FLT3-ITD-positive AML cellular damage such as apoptosis and serve as an alternate target for therapy [33]. ERK5 inhibitors significantly enhanced the anti-tumor effect of 5-FU, increased cell apoptosis, and inhibited the growth of tumors in colon cancer [34]. However, no study reported the role of MEK5 inhibitors in ccRCC. Our experiment applied MEK5 inhibitor BIX to ccRCC for the first time and demonstrated that BIX exhibited significant anti-tumor effects \textit{in-vitro and in-vivo} with excellent safety. Our results suggest that MEK5 could be a target for developing a new anti-cancer systemic treatment for ccRCC.

In summary, we proved that MEK5 expression aberrantly increased in ccRCC, which activated the mTOR signal pathway and regulated cell proliferation, cycle, migration, clone formation of ccRCC. Targeted inhibition of MEK5 has the potential for widespread clinical application in patients with ccRCC.

\textbf{Declarations}

\textbf{Funding}\(^\dagger\)

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

\textbf{Competing Interests}\(^\dagger\)

The authors have no relevant financial or non-financial interests to disclose.

\textbf{Author Contributions}\(^\dagger\)

Qi Chen, Yonghui Chen and Fangzhou Li conceived the research. Fangzhou Li, Xufeng Peng and Qi Chen designed the methodology. Fangzhou Li, Xufeng Peng, Jiale Zhou performed the experiments. Fangzhou Li wrote the original draft of the manuscript. Fangzhou Li and Xufeng Peng reviewed and edited the
manuscript. Yonghui Chen and Qi Chen supervised the study. All authors read and approved the final manuscript.

Data availability:

The datasets generated during the current study are not publicly available due to ethical restrictions, but are available from the corresponding author on reasonable request.

Ethical approval:

Human sample collection and the study protocol were approved by the Committee for the Ethical Review of Research, Renji Hospital, Shanghai Jiao Tong University School of Medicine.

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Figures

Figure 1

MEK5 is expressed abnormally in human ccRCC. a The expression of MEK5 mRNA in normal and tumor tissues were compared in Oncomine database. b QRT–PCR analysis of MEK5 mRNA levels in renal cancer tissues and corresponding adjacent tissue from 14 ccRCC patients. c Representative immunohistochemistry for MEK5 in human ccRCC samples.
Figure 2

MEK5 promoted proliferation, migration and colony formation. MEK5 regulates mTOR signaling pathway.

a Validation of MEK5 knockdown. MEK5 mRNA and protein levels were analyzed using qRT-PCR and western blot, respectively. b Effect of MEK5 knockdown on the proliferation of ccRCC cells. c Validation of MEK5 overexpression. MEK5 mRNA and protein levels were analyzed using qRT-PCR and western blot, respectively. d Effect of MEK5 overexpression on the proliferation of ccRCC cells. e Effect of MEK5
knockdown on the cell cycle of ccRCC cells. f Effect of MEK5 overexpression on the cell cycle of ccRCC cells. g Effect of MEK5 knockdown on the migration of ccRCC cells. h Effect of MEK5 overexpression on the migration of ccRCC cells. i Effect of MEK5 knockdown on the colony formation of ccRCC cells. j Effect of MEK5 overexpression on the colony formation of ccRCC cells. k Effect of MEK5 knockdown on Western blot analysis of involved mTOR signaling pathways and downstream targets. l Effect of MEK5 overexpression on Western blot analysis of involved mTOR signaling pathways and downstream targets. Cell proliferation was assessed by CCK-8 assay, cell migration was assessed by wound-healing assay, cell cycle was assessed by flow cytometry, cell colony formation was assessed by colony formation assay. Results are expressed as mean ± S.E.M. for samples in each category. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control.
Targeted inhibition of MEK5 has a good antitumor effect in vitro. a Effect of different concentrations of BIX (MEK5 inhibitor) on the proliferation of ccRCC cells. b Effect of different concentrations of BIX on the apoptosis of ccRCC cells. c Effect of different concentrations of BIX on the cell cycle of ccRCC cells. d Effect of different concentrations of BIX on the migration of ccRCC cells. e Effect of different concentrations of BIX on the colony formation of ccRCC cells. f Effect of different concentrations of BIX on Western blot analysis of involved mTOR signaling pathways and downstream targets. Cell proliferation was assessed by CCK-8 assay, cell migration was assessed by wound-healing assay, cell cycle and apoptosis were assessed by flow cytometry, cell colony formation was assessed by colony formation assay. Results are expressed as mean ± S.E.M. for samples in each category. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control.

**Figure 4**

Targeted inhibition of MEK5 has a good antitumor effect in murine models a Effect of MEK5 BIX on tumor growth. b Effect of MEK5 BIX on tumor volume. c Effect of MEK5 BIX on tumor murine weight. d Representative immunohistochemistry for MEK5 BIX. Results are expressed as mean ± S.E.M. for samples in each category. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control.