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Original Article

SLC18A3 promoted renal cancer development through acetylcholine/cAMP signaling

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Abstract: Renal cancer displays a high metastatic potential and a poor response to chemotherapy. However, the critical contributors to renal cancer development remain elusive. This study focused on acetylcholine (ACh) signaling. We identified the vesicular acetylcholine transporter (SLC18A3) that upregulates in patients with renal cancer. We further discovered that SLC18A3 enhanced the uptake of ACh, a classical neurotransmitter mediating synaptic transmission. The elevated ACh activated the protein kinase A (PKA)/cAMP-response element binding protein (CREB) pathway, which contributed to renal cancer cell proliferation and invasive migration. Consistently, SLC18A3 overexpression caused sustained tumor growth and increased lung metastases in A489-bearing mice. In summary, our study demonstrated that SLC18A3 contributed to cancer spread in an ACh/PKA/CREB-dependent manner, which may drive the design of efficacious treatment strategies.

Keywords: SLC18A3, acetylcholine, renal cancer, PKA, CREB

Introduction

Renal cancer is a highly malignant neoplasm of the urinary system, responsible for 102,000 annual deaths worldwide [1]. The current treatment options include surgical excisions and pharmaceutical interventions, such as chemotherapy, targeted therapy, and immunotherapy. However, their efficacy remains modest [2]. A notable proportion of patients with renal cancer experience chemoresistance and tumor metastasis, which pose major hurdles to successful therapy [3]. Once distant metastases develop, the five-year survival rates drop to less than 10% in patients with advanced renal cell carcinoma [4]. Therefore, healthcare professionals need to better understand renal cancer progression to devise treatment regimens for disease eradication.

The tumor develops in an intricate, multistep process, where the tumor microenvironment (TME) components are considered pivotal regulators. Besides the involvement of the immune cells and the extracellular matrix, recent evidence highlights that the neural system participates in the pathogenesis of malignancies [5-7]. To ensure their survival, tumor cells may drive neurogenesis and neural reprogramming using multiple mechanisms. In turn, infiltrating nerves have been proven to modify the behavior of tumor cells and their metastatic capacity [8]. The nervous system promotes tumor growth in part by releasing neurotransmitters, including ACh. ACh is a ubiquitous signaling molecule involved in multiple pathological processes, including infections, autoimmune disorders, and neoplasia [9]. Upon synthesis, ACh is packaged into vesicles by the vesicular acetylcholine transporter (VAChT) and exocytosed into the extracellular milieu, where ACh binds to its cognate receptors and then orchestrates downstream pathways. Several components of the ACh signaling contributed to tumor advancement through complex functions [10]. For instance, in gastric cancer, ACh induced the expression of epithelial-mesenchymal transition (EMT) markers, highlighting the role of ACh in malignant conversion and metastatic seeding [11]. Ukegawa et al. reported that the activation of the m3 muscarinic acetylcholine receptor (m3 mAChR) stimulated the p21Ras-ERK pathway in a manner dependent on EGFR, thus enhancing cell growth in colon cancer [12].

What is more, clinical evidence shows that VAChT upregulation correlated with an increased risk and recurrence in lung adenocarcinoma [13]. Nevertheless, few studies have elucidated the underlying mechanisms of ACh signaling in tumorigenesis and the progression of renal cancer.

In this study, we shed light on the ACh transporter and found that the augmented expression of SLC18A3 (also known as VAChT) portended a grim prognosis in patients with renal cancer. We provided evidence that SLC18A3 accelerated cell proliferation and migration by enhancing ACh uptake in patients with renal cancer. Meanwhile, data from *in vivo* experiments showed that SLC18A3 overexpression caused an increase in tumor size and the number of metastases. Our study further unveiled the molecular mechanism of SLC18A3-induced tumor progression, which is dependent on the PKA/CREB pathway. Importantly, the aberrant expression of ACh/PKA/CREB correlated with tumor development in the clinic. This finding may help devise therapeutic strategies for renal cancer.

Materials and methods

Cell culture and reagents

Human renal cell carcinoma (RCC) cell lines (A489 and 786-0) were purchased from the American Type Culture Collection (ATCC, USA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 complete culture medium (Thermo Fisher, USA), containing 10% fetal bovine serum (FBS, Gibco, USA) at an atmosphere of 5% CO_2. SLC18A3-overexpressing A489 and 786-0 cell lines were established using COBIOER (China). To regulate the expression level of the SLC18A3 gene, The pCMV plasmid was transfected using Lipofect8000 (Thermo Fisher, USA) to induce gene overexpression. The pCMV-SLC18A3-3 × FLAG vectors were obtained from COBIOER (China). SLC18A3-overexpressing A489 and 786-0 cell lines were determined using western blotting. ACh and H89 2HCl, a PKA inhibitor, were purchased from Selleck (USA).

Clinical specimens

Twenty-four renal tumor tissues were obtained from Shaanxi Provincial People's Hospital and divided into metastatic and non-metastatic groups (n = 12 in each group) according to the follow-up visits (5 years). The Ethical Committee of Shaanxi Provincial People's Hospital approved the experimental protocols. The transcriptome and patients' information (526 renal tumor tissues and 72 normal tissues) were obtained from https://xenabrowser.net/. T-test was performed using GraphPad 6.0 software.

Cell proliferation

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay kit according to the manufacturer's instructions (Solarbio, China). Briefly, A489 and 786-0 cells were pretreated with PBS, ACh, or H89 2HCl and transferred onto 96-well plates. After 24, 48, and 72 hours, CCK-8 solution (10 μl) was added to 96-well plates, which were cultured for another hours. The cell density was determined by measuring the absorbance at 450 nm using a microplate reader (Thermo Fisher, USA).

Transwell assay

A489 and 786-0 cells were pre-treated with ACh or H89 2HCl. Subsequently, 5×10^4 A489 or 786-0 cells were seeded in a Transwell chamber (8 μm, Thermo Fisher, USA) containing a 300 μl culture medium (10% FBS). One ml FBS-free culture medium was added to a 24-well plate. Twenty-four hours later, the chamber was fixed with paraformaldehyde and stained with crystal violet. Migrating cell numbers were determined under the microscope (Leica, Germany).

Enzyme-linked immunosorbent assay (ELISA)

Total and intracellular ACh quantification were conducted using a human acetylcholine ELISA kit according to the manufacturer's instructions (Sangon, China). For the total ACh quantification, 5 mg of tumor tissues were lysed by RIPA lysis buffer (Solarbio, China). For the intracellular ACh quantification, 2×10^6 cells were collected, washed with PBS, and lysed. All samples were centrifuged at 3000 rpm for 5 min at 4°C and then immediately analyzed to determine ACh concentration.

Western blotting

Equal amounts of proteins (20 μg) were loaded into each lane and separated using electropho-

resis. Subsequently, proteins were transferred onto a PVDF membrane and blocked with a blocking buffer (5% bovine serum albumin). One hour later, samples were incubated overnight at 4°C with the following primary antibodies: anti-SLC18A3 (1:1000, NB100-91348, Novus Biologicals, USA), anti-PKA (1:1000, ab75991, Abcam, UK), and anti-phosphorylated CREB (1:1000, ab32096, Abcam, UK). Then, the blots were incubated for one hour at room temperature with horse radish peroxidase-(HRP-) conjugated secondary antibodies and visualized.

Immunohistochemistry and immunofluorescence

Paraffin sections were dewaxed, followed by a sodium citrate antigen retrieval. Subsequently, sections were blocked with a blocking buffer (5% bovine serum albumin) for 30 minutes at room temperature and incubated overnight at 4°C with the following primary antibodies: anti-SLC18A3 (1:1000, NB100-91348, Novus Biologicals, USA), anti-PKA (1:1000, ab75991, Abcam, UK), and anti-phosphorylated CREB (1:1000, ab32096, Abcam, UK). For the immunofluorescence, the samples were incubated with goat anti-rabbit secondary antibodies (Thermo Fisher, USA) and stained with 4',6-diamidino-2-phenylindole (DAPI) (Solarbio, China). For the immunohistochemistry, the samples were incubated with HRP-conjugated secondary antibodies and visualized. Protein expression was quantified using Image Plus Pro 6.0 software.

Xenograft mice model

Female 6~8 weeks old NOD-SCID mice were obtained from Huafukang (China) and maintained in a specific-pathogen-free (SPF) room. Mice were subcutaneously injected with 2×10^6 vector or SLC18A3-overexpressing A489 cells. Tumor volume was recorded every day (n = 6 in each group). The tumor volume was calculated as follows: tumor volume = length × width^2/2. After 40 days, the mice were sacrificed, and the tumor tissues were collected for protein evaluation assay. For the pulmonary metastasis assay, 1×10^6 vector or SLC18A3-overexpressing A489 cells were injected into the tail veins of NOD-SCID mice (n = 6 in each group). After 40 days, mice were sacrificed, and pulmonary metastatic tumor nodules were assessed using hematoxylin & eosin (H&E) staining and counted. All animal studies were conducted following the Public Health Service Policy and complied with the WHO guidelines for the humane use and care of animals. The Animal Ethics Committee of Shaanxi Provincial People's Hospital monitored all animal protocols.

Statistical analysis

Data were presented as mean ± SEM, and each experiment was performed at three independent times. Differences were evaluated using a T-test for two groups and one-way ANOVA for three or more groups using Graphpad 6.0 software. The Kaplan-Meier survival analysis was used to evaluate the overall survival (*P < 0.05; **P < 0.01; ***P < 0.001; ns: no significant difference).

Results

SLC18A3 was upregulated in renal cancer

Seeking to elucidate the mechanism of renal cancer development, we compared the transcriptome expression in 526 renal tumor tissues with that in 72 normal tissues using the TCGA database. One hundred thirty-four differentially expressed genes ($\log_2$ fold change > 5; P < 0.05) were identified in the tumor and normal tissues (69 genes were downregulated and 65, upregulated; **Figure 1A**). To further determine the role of differentially expressed genes in tumor progression, Cox proportional hazards analysis was performed for the top 60 differentially expressed genes (P < 0.05; top 60 upregulated and downregulated genes), and 15 genes (P < 0.05 in survival analysis) were identified as major regulators that influence the overall survival in patients with renal cancer (**Figure 1B**). In fact, among the 15 genes, a series of oncogenes were involved in tumor progression, including TERT2, NPTX2, IL20RB, and OR2A4. Intriguingly, we found that SLC18A3 displayed a high hazard ratio (1.306; P < 0.001), while its role in regulating tumor progression remains elusive. Therefore, we compared SLC18A3 expression in 526 renal tumor tissues with that in 72 normal tissues and found that it was markedly upregulated in tumor tissues (**Figure 1C**). To further validate this result, using immunohistochemistry, we examined SLC18A3 expression at the protein

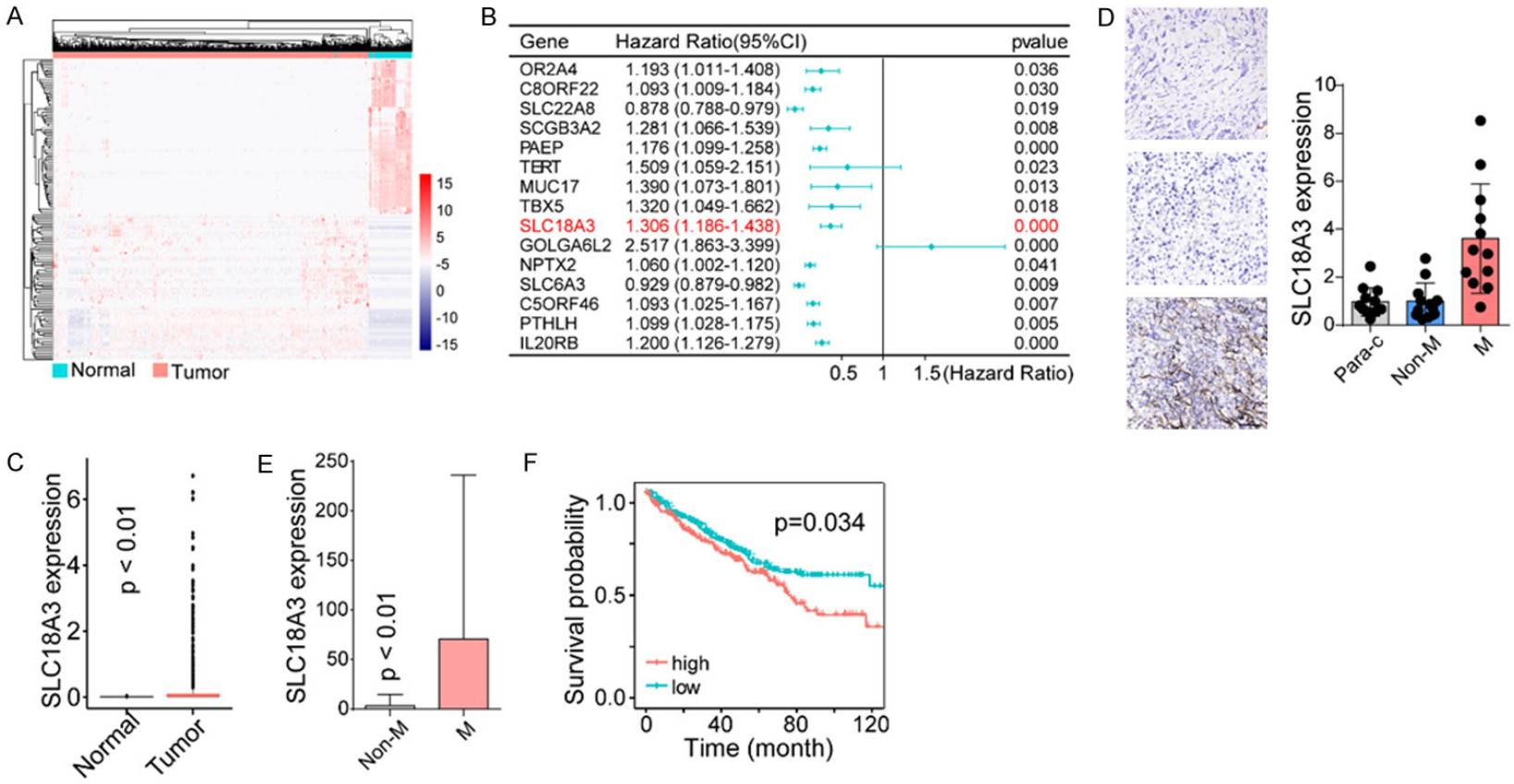

Figure 1. SLC18A3 was upregulated in renal cancer. A. Heat map of 134 differentially expressed genes (log2 fold change > 5, P < 0.05) were identified between 526 renal tumor and 72 normal tissues (downregulated 69, upregulated 65). B. COX proportional-hazards analysis of 15 genes in 526 patients with renal cancer. C. The transcriptome expression of SLC18A3 in 526 renal tumor and 72 normal tissues. D. Immunohistochemistry of SLC18A3 in 12 para-carcinoma tissues and 24 renal tumor tissues from non-metastatic (n = 12) and metastatic (n = 12) patients. SLC18A3 expression was quantified and the scale bar was 50 μm. E. The transcriptome expression of SLC18A3 in tumor tissues from 374 non-metastatic and 61 metastatic patients with renal cancer. F. Overall survival of 526 renal cancer patients divided into high SLC18A3 (n = 261) and low SLC18A3 (n = 261) groups, analyzed by utilizing TCGA databases.

level in 24 tumor tissues, which were divided into para-carcinoma tissues, non-metastatic and metastatic groups according to follow-up visits. Accordingly, we observed a remarkable increase in SLC18A3 expression in the metastatic patients compared with the non-metastatic or para-carcinoma ones (**Figure 1D**). Elevated SLC18A3 expression was also found in metastatic patients derived from TCGA database (**Figure 1E**). Because of the strong expression of SLC18A3 in renal tumor tissues, especially in the metastatic group, we next assessed whether SLC18A3 influenced the overall survival of patients. Thus, we divided 522 patients with renal cancer from the TCGA database into high (n = 261) and low (n = 261) SLC18A3 groups. Consistently, the "high SLC18A3" group displayed a remarkably shortened survival time compared with the "low SLC18A3" group (**Figure 1F**). Those results suggested that SLC18A3 might serve as a potential prognostic marker for renal cancer and play a role in regulating renal cancer progression.

SLC18A3 facilitated ACh uptake to regulate cell behavior

Motivated by the previously mentioned results that high expression of SLC18A3 correlated with a poor prognosis in patients with renal cancer, we next sought to explore the mechanism of renal cancer progression induced by SLC18A3. To do this, first, we established SLC18A3 overexpression in renal cancer cell lines A489 and 786-0 (**Figure 2A**). Subsequently, we conducted CCK-8 and Transwell assay to evaluate the influence of SLC18A3 on cell proliferation and migration. However, no obvious difference was observed between SLC18A3 overexpression and vector group (**Figure 2B** and **2C**). SLC18A3, a member of the vesicular ACh transporter family, encodes a transmembrane protein that transports ACh into intracellular presynaptic secretory vesicles. Notably, ACh is an ester of acetic acid and choline, which acts as a neurotransmitter and has been reported to promotes lung carcinoma cell proliferation [14]. Thus, we supposed that high expression of SLC18A3 might increase ACh uptake efficiency in tumor cells, resulting in proliferative characteristics induced by intracellular ACh. To validate our hypothesis, we treated the vector and SLC18A3-overexpressing A489 and 786-0 cells for 48 hours with ACh. Then, we examined the intracellular ACh concentration using an ACh ELISA kit. As expected, increased intracellular ACh was observed in SLC18A3-overexpressing A489 and 786-0 cells (**Figure 2D**). More importantly, ACh enhanced cell proliferation and migration in SLC18A3-overexpressing A489 and 786-0 cells, while limited pro-tumor effects were observed in vector A489 and 786-0 cells (**Figure 2E** and **2F**). Subsequently, we examined the expression of ACh in 24 clinical renal tumor tissues. Intriguingly, both the non-metastatic and metastatic groups displayed similar ACh concentrations in tumor tissues (**Figure 2G**), while tumor cells from the metastatic group showed increased intracellular ACh accumulation (**Figure 2H**) compared with the non-metastatic group. Given the higher SLC18A3 expression in the metastatic group and the pro-tumor effects induced by the SCL18A3/ACh axis *in vitro*, we concluded that SLC18A3 served as an ACh transporter, promoting ACh uptake in renal cancer cells, thereby causing sustained tumor growth and metastasis *in vivo*.

ACh mediated PKA/CREB signaling activation

To elucidate the mechanism underlying ACh-induced cell proliferation/migration, we next divided 526 patients with renal cancer into a "high SLC18A3" group (n = 263) and a "low SLC18A3" group (n = 263). The mRNA expression of those patients with renal cancer was analyzed using the TCGA database. Four hundred and forty-nine differentially expressed genes (294 upregulated and 155 downregulated) were identified (**Figure 3A**). Next, utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, the major pathways involved with SLC18A3 were determined (**Figure 3B**). In agreement with our aforementioned results, SLC18A3 was involved in neuroactive ligand-receptor interaction (ACh) associated signaling. More importantly, SLC18A3 also participated in modulating the cAMP signaling pathway, a crucial driver of tumor development. To determine the role of cAMP signaling in regulating renal cancer progression, the major cAMP molecule PKA was detected in A489 and 786-0 cells using western blotting. Notably, SLC18A3-overexpressing A489 and 786-0 cells treated with ACh exhibited an obvious upregulation of PKA (**Figure 3C**), suggesting that intracellular ACh mediated the activation of cAMP signaling in renal cancer. Compelling studies have demonstrated that PKA could

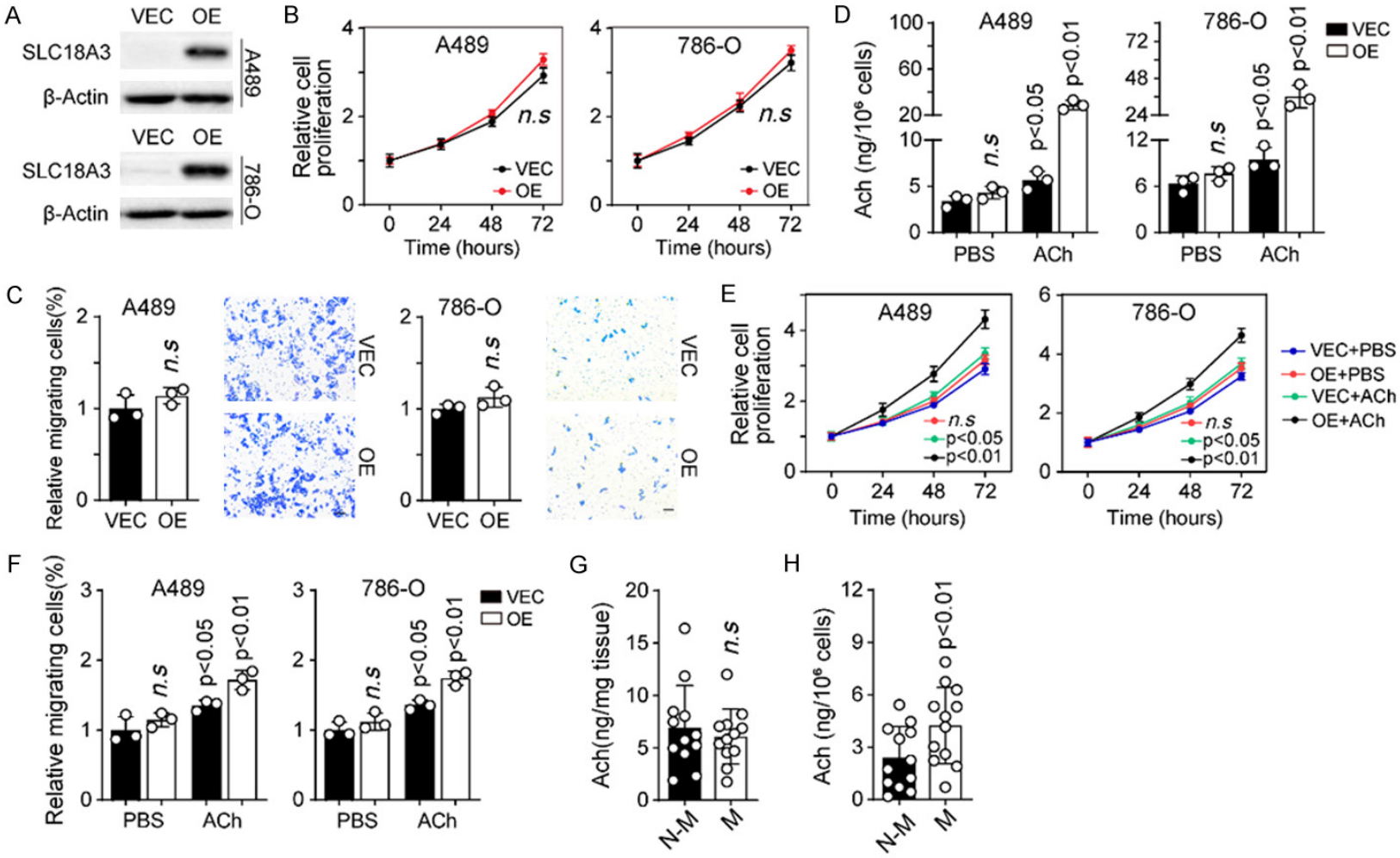

Figure 2. SLC18A3 facilitated ACh uptake to regulate cell behavior. (A) Western blotting of SLC18A3 in vector and SLC18A3 overexpression A489/786-O cells. (B) Cell proliferation of vector and SLC18A3 overexpression A489/786-O cells, determined by CCK-8 analysis. (C) Relative migrating cells of vector and SLC18A3 overexpression A489/786-O cells, determined by Transwell assay. The scale bar was 50 μm. (D) Intracellular ACh quantification in vector and SLC18A3 overexpression A489/786-O cells treated with ACh (5 μM, 48 hours) or not. (E) Cell proliferation of vector and SLC18A3 overexpression A489/786-O cells treated with ACh (5 μM, 48 hours) or not, determined by CCK-8 assay. (F) Relative migration cells of vector and SLC18A3 overexpression A489/786-O cells treated with ACh (5 μM, 48 hours) or not, determined by Transwell assay. (G) ACh quantification in 24 tumor tissues from non-metastatic (n = 12) and metastatic (n = 12) patients. (H) Tumor cells were isolated from 24 tissues in (G), and intracellular ACh were quantified.

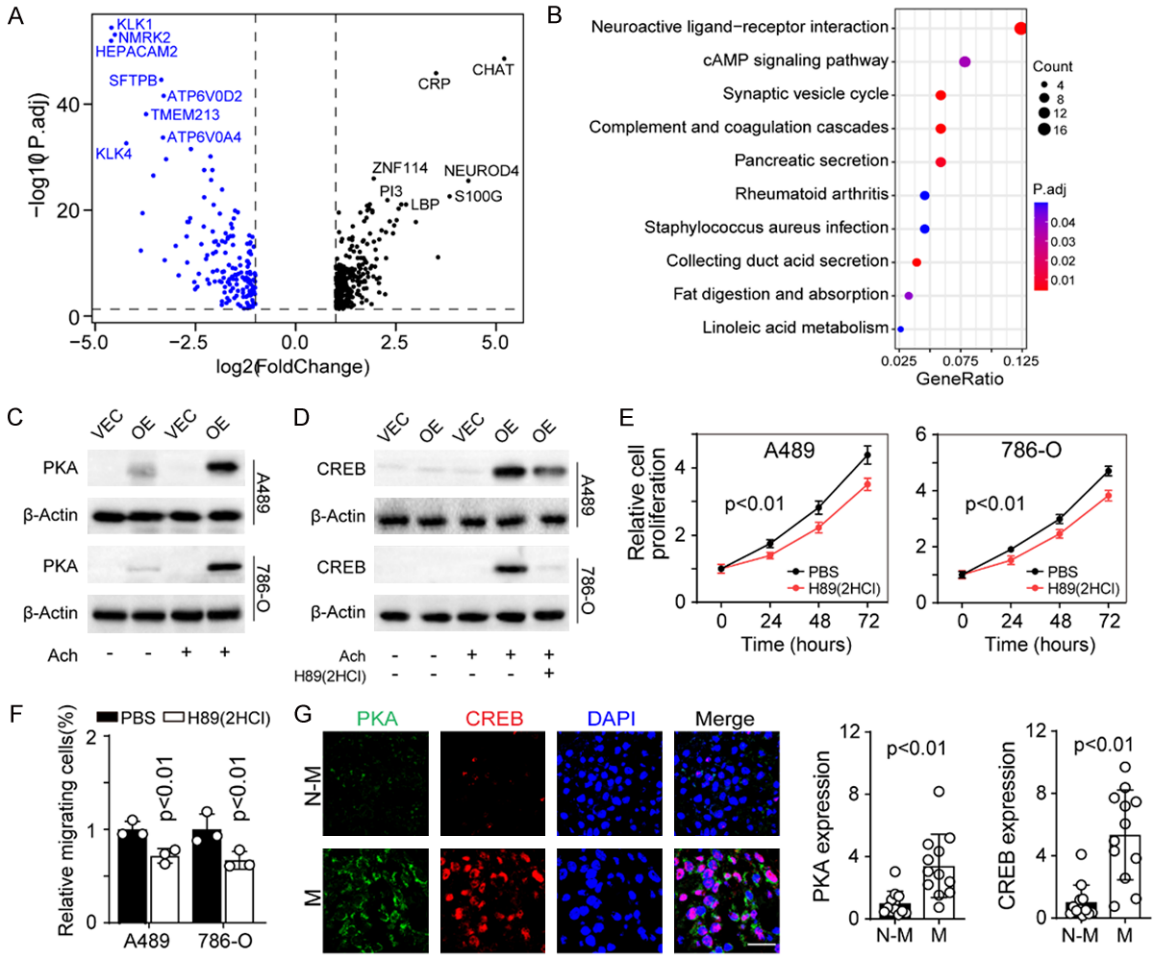

Figure 3. ACh mediated PKA/CREB signaling activation. (A) 526 renal cancer patients were divided into high SLC18A group (n = 263) and low SLC18A3 group (n = 263), and differentially expression genes were determined. Volcano plot of 449 significant differentially expressed genes identified through differential analysis with 294 genes up-regulated and 155 genes downregulated, respectively. (B) KEGG enrichment analysis was conducted in 526 renal cancer patients, divided into high SLC18A group (n = 263) and low SLC18A3 group (n = 263). (C) Western blotting of PKA in vector and SLC18A3 overexpression A489/786-O cells treated with ACh (5 μM, 48 hours) or not. (D) Western blotting of CREB in vector and SLC18A3 overexpression A489/786-O cells treated with ACh (5 μM, 48 hours), H89 (2HCl) (10 nM, 48 hours) or not. (E and F) SLC18A3 overexpression A489/786-O cells were treated with ACh (5 μM) combining H89 (2HCl) (10 nM) or not. Cell proliferation (E) and relative migration cells (F) were determined using CCK-8 and Transwell assay. (G) Immunofluorescence of PKA and CREB in 24 tumor tissues from non-metastatic (n = 12) and metastatic (n = 12) patients. Protein expression was quantified and the scale bar was 50 μm.

mediate the CREB upregulation to stimulate cell proliferation and migration [14, 15]. Thus, we further examined the expression of CREB in renal cancer cells. As expected, increased CREB was observed in SLC18A3-overexpressing A489 and 786-O cells treated with ACh, while treatment with H89 2HCl, a PKA inhibitor, suppressed CREB upregulation (**Figure 3D**). Those results suggested that SCL18A3/ACh axis mediated PKA/CREB signaling activation in renal cancer. Next, to further confirm the role of PKA/CREB signaling in regulating renal cancer development, we added H89 2HCl to SLC18A3-overexpressing A489 and 786-O cells treated with ACh and assessed cell proliferation and migration. As expected, treatment with H89 2HCl suppressed cell proliferation (**Figure 3E**) and migration (**Figure 3F**), revealing that the SLC18A3/ACh axis promoted renal cancer development through PKA/CREB signaling. Moreover, using immunofluorescence, we examined PKA/CREB expression in renal tumor tissues obtained from non-metastatic and metastatic patients. Upregulated PKA and CREB (**Figure**

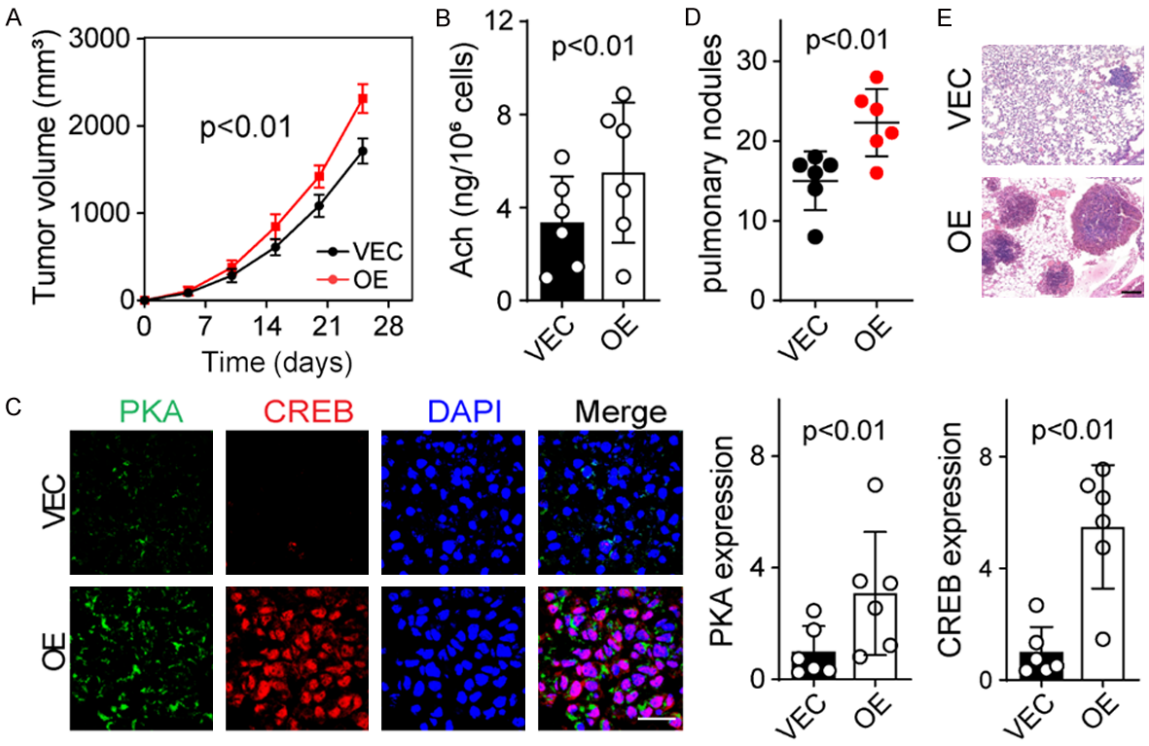

Figure 4. SLC18A3 promoted renal tumor progression in vivo. (A) Tumor volume of subcutaneous vector/SLC18A3 overexpression A489 bearing mice. (B) Intracellular ACh quantification of tumor cells from vector/SLC18A3 overexpression A489 bearing mice. (C) Immunofluorescence of PKA and CREB in tumor tissues from vector/SLC18A3 overexpression A489 bearing mice. Protein expression was quantified and the scale bar was 50 μm. (D) Metastatic pulmonary tumor nodule quantification in mice injected with vector or SLC18A3 overexpression A498 cells (1×10^6, day 40). (E) H&E staining of lung tissues in (D). The scale bar was 200 μm.

3G) were observed in tumor tissues obtained from metastatic patients compared with non-metastatic ones. Collectively, those results suggested that intracellular ACh mediated PKA/CREB signaling activation to promote renal cancer progression.

SLC18A3 promoted renal tumor progression in vivo

Our *in vitro* findings have suggested that SLC18A3/ACh promoted cell proliferation. We became interested in evaluating the influence of SLC18A3 on tumor progression *in vivo*. Vector and SLC18A3-overexpressing A489 cells were subcutaneously inoculated into NOD-SCID mice. Intriguingly, we found a rapid tumor growth trend in mice bearing SLC18A3-overexpressing A489 cells compared with the mice bearing vector cells (**Figure 4A**). In line with the *in vitro* results, tumor cells isolated from mice bearing SLC18A3-overexpressing A489 cells revealed a higher intracellular ACh level compared with mice bearing vector cells (**Figure 4B**), suggesting that SLC18A3 promoted ACh uptake of tumor cells *in vivo*. Similarly, at the protein level, PKA and CREB were upregulated in mice bearing SLC18A3-overexpressing A489 as evidenced by immunofluorescence (**Figure 4C**). Subsequently, we further evaluated the effects of SLC18A3 on the pulmonary metastasis of renal cancer cells. We injected vector cells or SLC18A3-overexpressing A498 cells (1×10^6 cells) into the tail vein of mice. After 40 days, mice were sacrificed, and consecutive sections of lung tissues were subjected to hematoxylin & eosin staining. We examined and counted all the metastatic tumor nodules to assess pulmonary metastasis. In agreement with the *in vitro* Transwell assay, cells with SLC18A3 overexpression formed more metastatic tumor nodules than vector cells (**Figure 4D** and **4E**), indicating a pro-metastasis role of SLC18A3 in renal cancer. Collectively, our experiments proved that SLC18A3 contributed to the acceleration of renal cancer cell prolifer-

ation and migration, resulting in sustained tumor progression *in vivo*.

Discussion

Cancer cells take over the microenvironment to maximize their survival advantages. As a common feature of the TME, the nerves have been intimately linked to the malignant etiology of tumors [16]. This study complemented the previous findings underscoring that SLC18A3 participated in modulating the aggressive traits of renal cancer cells in an ACh-dependent manner. Meanwhile, we showed that high SLC18A3/ACh levels were related to a greater tumor burden in patients with renal cancer. By connecting ACh signaling with tumor progression, our work opens new perspectives for future renal cancer research.

ACh, as a regulator of tumor behaviors, has long been under intense investigation. Research has converged to suggest that ACh could be auto-synthesized and released as a facilitator in several tumor types [17, 18]. ACh was proved to stimulate the tumor's sphere formation and increase the expression of stemness markers in gastric cancer, indicating its role in maintaining a stem cell phenotype [19]. Additionally, extensive literature stated that enzymes and receptors involved in ACh signaling contributed to the aggressive traits of tumor cells, while ACh transporters have received less attention [20]. However, evidence showed that SLC18A3 was robustly expressed in clinical samples from patients with lung cancer [21]. Moreover, the blockade of the vesicular transporter activity of SLC18A3 showed the growth-inhibitory effects on lung cancer *in vitro* and *in vivo* [22]. In a recent investigation on head and neck cancer, SLC18A3 was identified as part of the gene expression signature associated with angiolymphatic invasion, which predicted disease relapse and unfavorable survival [22, 23]. Our study revealed an elevated expression of SLC18A3 in renal tumor tissues, which motivated us to postulate that SLC18A3 may modulate the development of renal cancer. On this basis, we demonstrated that SLC18A3 promoted cell proliferation and migration, thus fueling renal cancer growth. We further attributed the pro-tumoral properties of SLC18A3 to the enhancement of ACh uptake. Our study reported for the first time that SLC18A3, the vesicular transporter responsible for ACh packaging, contributed to renal cancer progression.

cAMP, the first discovered second messenger, is produced in response to the stimulation of a plethora of hormones and neurotransmitters. It has been well documented that cAMP modulates the transcription of various target genes, mainly through PKA and its downstream effectors, to orchestrate correct cellular responses [24]. cAMP-PKA signaling is pervasively activated in human neoplasms, and uncontrolled PKA activation is frequently reported to contribute to neoplastic transformation, angiogenesis, and apoptotic events, ultimately dictating the fate of a tumor [25]. PKA is, therefore, exploited as a relevant biological target, and inhibitors of PKA are currently being applied singly or in combination with a medication to effectively treat cancer [26]. In line with the aforesaid findings, our study confirmed that PKA endowed renal cancer cells with enhanced ability of proliferation and migration, which could be reversed by PKA blockade. Importantly, we emphasized the implication of PKA signaling in SLC18A3-induced renal cancer progression, as indicated by the upregulation of PKA in SLC18A3-overexpressing cells. KEGG enrichment analysis also demonstrated that SLC18A3 modulates the cAMP signaling pathway. The pro-tumor role of PKA involves multiple substrates. Tonucci and colleagues found that PKA facilitated tumor metastasis via the phosphorylation of CDC42 interacting protein 4 (CIP4), an orchestrator of membrane deformation and actin polymerization [27]. FAK was another PKA target that conferred invasive capacity to prostate cancer cells [28]. We proposed that ACh treatment significantly augmented PKA expression in renal cancer cells and activated CREB, a tumor co-conspirator participating in cell survival and glucose metabolism. Our study further validated that SLC18A3 delivered a signal through the PKA/CREB pathway, leading to sustained tumor growth and increased metastatic lesions in murine xenograft models. Based on the aforementioned results, our study underscored the relevance of SLC18A3 in renal cancer progression. First, our analysis of the TCGA database suggested that SLC18A3 expression indicated renal cancer, with high values predicting unfavorable outcomes. Second, we provided evidence that SLC18A3 promoted the proliferative properties and migratory capacity

of renal cancer cell lines in mice models. Third, our experiment indicated a novel pathway whereby SLC18A3 stimulated tumor growth via ACh/PKA/CREB signaling. Fourth, we found that the aberrant expression of ACh/PKA/CREB was correlated with metastasis in the clinic, which might provide potential biomarkers to monitor renal cancer progression.

Conclusion

We demonstrated that SLC18A3 signaled through the ACh/PKA/CREB pathway, thus facilitating cell proliferation and metastasis in renal cancer. Our findings raise the tantalizing possibility that targeting the autonomic nervous system may be a feasible treatment modality for renal cancer.

Acknowledgements

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The study was approved by the ethical committee of Shaanxi Provincial People's Hospital. Informed consent was obtained from all subjects involved in this study. All animal experiments in this study were approved by the Shaanxi Provincial People's Hospital, in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and all methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

Disclosure of conflict of interest

None.

Abbreviations

SLC18A3, vesicular acetylcholine transporter; ACh, acetylcholine; PKA, Protein kinase A; CREB, cAMP-response element binding protein; TME, tumor microenvironment; VAChT, vesicular acetylcholine transporter; EMT, epithelial-mesenchymal transition; ATCC, American Type Culture Collection; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; ELISA, Enzyme-linked immunosorbent assay; SPF, Specific Pathogen Free; CIP4, CDC42 interacting protein 4.

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