Identification of ACSL4 as a biomarker and contributor of ferroptosis in clear cell renal cell carcinoma

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Primary research

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Abstract

Background

ACSL4 has been reported to be related to tumor genesis and involved in the processes of ferroptosis. However, the expression levels and prognostic value of ACSL4 in clear cell renal cell carcinoma (ccRCC) remain unclear.

Methods

The Oncomine and TCGA databases were used to predict the expression of ACSL4 mRNA in ccRCC and its association with ccRCC prognosis. The expression levels of ACSL4 were determined in human RCC tissues by real-time PCR. Kaplan-Meier curves were used to analyze the diagnostic and prognostic significance of ACSL4 in ccRCC. A ferroptosis inducer (erastin) was used to investigate the effects of ACSL4 on ferroptosis in ccRCC cell lines.

Results

The expression level of ACSL4 was significantly down-regulated in ccRCC tissues ($P < 0.001$), which was consistent with the analysis of the Oncomine and TCGA database. Then, immunohistochemical results demonstrated that the ACSL4 was weak or not detected in ccRCC tissues than that in normal tissues. ACSL4 differential expression level was significantly related to gender, ccRCC subtypes, nodal invasion, tumor grade and cancer stages (all $P < 0.001$). Survival analysis revealed that overall survival was favorable in ccRCC patients with ACSL4 high expression ($P = 0.014$). Overexpression of ACSL4 by gene transfection restores ferroptosis sensitization in cancer cells, whereas suppression of ACSL4 expression by RNAi increases ferroptosis resistance. Mechanically, protein ubiquitination may be involved in ACSL4-mediated ferroptosis.

Conclusions

As a monitor and contributor of ferroptosis, ACSL4 was decreased in ccRCC and served as a useful diagnostic and prognostic biomarker, which will be a new potential therapeutic target for ccRCC.

Background

As one of the most common malignant neoplasms of the urinary system, renal cell carcinoma (RCC) accounts for 2–3% of malignancies in adults. Clear cell RCC (ccRCC), the most common subtype of RCC, generally has the characteristics of high metastasis risk and poor response to radiotherapy and chemotherapy. It is noteworthy that 30% of patients with ccRCC present with localized progression and distant metastasis when clinically diagnosed, and this represents their main cause of death [1]. Therefore,

it is helpful to develop diagnostic and therapeutic targets through identification of genes which are differentially expressed in ccRCC.

ACSL4, also known as FACL4, was initially identified as its mutation in non-specific X-linked mental retardation in 2002 [2]. Maloberti et al. found that ACSL4 possessed a substrate preference for eicosapentaenoic acid and arachidonic acid (AA). More interestingly, the level of intracellular ACSL4 protein can be altered in turn by the amounts of free AA [2]. So far, it has been proven that the ACSL4 dysregulation is related to a great number of diseases including diabetes [3], acute kidney injury [4], and malignant tumors [5]. Intrinsic or acquired resistance to apoptosis is a hallmark of cancer. Thus, induction of non-apoptotic forms of regulated cell death has become an emerging anticancer strategy for therapeutics [6]. Ferroptosis is a recently identified, non-apoptotic, regulated cell death first observed in cancer cells with oncogenic Ras mutation [7]. Recent studies have indicated that the dysregulation of ACSL4 is closely related to ferroptosis [8]. Knockdown of ACSL4 inhibited erastin-induced apoptosis in HepG2 and HL60 cells, and ACSL4 overexpression restored the sensitivity of LNCaP and K562 cells to the ferroptosis inducer Erastin [6]. Therefore, the expression levels of ACSL4 may be related to the development of ccRCC by mediating ferroptosis. However, the expression pattern and functional role of ACSL4 in ccRCC have not been examined. Therefore, in the present study, ACSL4 expression in ccRCC and its relationships with patients' clinicopathological factors and prognosis were investigated, as well as the role of ACSL4 on ferroptosis by performing experimental and bioinformatic analyses.

Material And Methods

Bioinformatics analysis of ACSL4

First, the Oncomine database (<https://www.oncomine.org/>) was used to predict the ACSL4 mRNA expression levels in ccRCC and normal tissues. Then the Cancer Genome Atlas (TCGA) KIRC database was used to predict the relationship between expression levels of ACSL4 mRNA and clinical prognosis of ccRCC patients from UALCAN (<http://ualcan.path.uab.edu>). In The Human Protein Atlas (<https://www.proteinatlas.org>), direct comparison of ACSL4 protein expression between human normal and ccRCC tissues was performed by immunohistochemistry image. The prognostic value of ACSL4 mRNA expression was evaluated using an online database, Kaplan-Meier Plotter (www.kmplot.com). The TCGA_KIRC dataset was selected for ACSL4 co-expression genes analysis using cBioPortal (<http://www.cbioportal.org/>). Functions of ACSL4 were analyzed by GO and KEGG in the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/summary.jsp>).

Human ccRCC tissue samples

A total of 16 patients diagnosed as ccRCC between 2018 and 2019 at the Sun Yat-sen University cancer center were included in this study, whose cancer tissues and adjacent tissues (5 cm away from the edge of the tumor tissues) were collected for subsequent qPCR assays. These patients did not receive any radiotherapy, chemotherapy, or other adjuvant therapy before surgery. This study was approved by the

Academic Committee of Sun Yat-Sen University cancer center, and all patients provided informed consent.

Cell culture

786-O, 769-P, A498, and RCC4 were purchased from the American Type Culture Collection (Manassas, VA, USA). We used DMEM high glucose medium with 10% fetal bovine serum, and 100 U/ ml of penicillin/streptomycin in a humidified incubator with 5% CO₂ and 37 °C culture environment.

Cell Proliferation

Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the ability of cell proliferation according to manufacturer's instructions.

Western blot assays

Concisely, the tissues or cells were lysed by RIPA buffer and the concentration of the proteins was determined by a BCA kit (Beyotime Institute of Biotechnology). A total of 30 µg total proteins from the tissues or cells were subjected to SDS-PAGE and the proteins were transferred to a PVDF membrane (Millipore, Bedford, MA). The PVDF membranes were blocked in 5% skim milk for 1 hour at room temperature and then incubated with primary antibody overnight at 4 °C. After that, the PVDF membranes were washed in PBST 3 times and then incubated with secondary antibody for 2 hour at room temperature. Finally, the signals were visualized via using enhanced or super chemiluminescence (Pierce, Rockford, IL, USA) and by exposure to X-ray films.

Quantitative real time polymerase chain reaction (qPCR) analysis

First-strand cDNA synthesis was carried out by using a Reverse Transcription System Kit from OriGene Technologies according to the manufacturer's instructions. cDNA from cell samples was amplified with specific primers (ACSL4: 5'- GCTATCTCCTCAGACACACCGA-3' and 5'-AGGTGCTCCAACTCTGCCAGTA-3') and the data was normalized to actin RNA (5'-CACCATTGGCAATGAGCGGTTC - 3' and 5'-AGGTCTTTGCGGATGTCCACGT - 3').

RNAi and gene transfection

The human ACSL4-shRNA was obtained from Sigma. Human ACSL4-cDNA was purchased from OriGene Technologies. Transfections were performed with Lentivirus Transduction System (Sigma) or Lipofectamine™ 3000 (Invitrogen) according the manufacturer's instructions.

Iron assay

Intracellular or mitochondrial ferrous iron (Fe²⁺) level was determined using the iron assay kit purchased from Sigma Aldrich (Milwaukee, WI, USA) according to the manufacturer's instructions.

Lipid ROS assays

For lipid ROS, cells were treated as indicated, and then trypsinized and resuspended in medium plus 10% FBS. Then, 10 μ M of C11-BODIPY (Thermo Fisher) was added and the samples were incubated for 30 min at 37 °C, 5% CO₂, and protected from light. Excess C11- BODIPY was removed by washing the cells with PBS twice. The fluorescence of C11-BODIPY581 = 591 was measured by the simultaneous acquisition of green (484/510 nm) and red signals (581/610 nm) using a flow cytometer.

Statistical analysis

The statistical analysis software used in this study were SPSS 22.0 (IBM SPSS, Chicago, IL) and GraphPad Prism 6.0 (GraphPad Software, Inc., USA). Statistical analysis Data are expressed as means $\pm$ SD. Significance of differences between groups was determined using two-tailed Student's t-test or ANOVA LSD test. A p-value < 0.05 was considered significant.

Results

ACSL4 Expression is Down-regulated in ccRCC

We first used the Oncomine database to find that ACSL4 mRNA expression level was significantly lower in ccRCC tissues than that in normal tissues (Fig. 1). The results were consistent with those from the TCGA database (Fig. 3A). Tissues qPCR was performed to detect ACSL4 expression in surgical ccRCC tumor tissues and normal kidney tissues. The relative expression of ACSL4 significantly decreased in tumor tissues (n = 16) compared with the corresponding normal counterparts (n = 16) (P < 0.0001, Fig. 2A). Subsequently, we detect the protein expression of ACSL4 by reviewing the Human Protein Atlas dataset (<https://www.proteinatlas.org/>). Moderate or strong ACSL4 staining was observed in normal kidney tubules cells (Fig. 2B) and ACSL4 positive expression rate was 72.7% (8/11). Conversely, almost no ACSL4 staining or very weak staining was observed most malignant tissues, and staining was mainly localized in the cell membrane and cytoplasm (Fig. 2C-D).

Low ACSL4 mRNA Expression is Correlated with Malignant Clinicopathological Features in ccRCC patients

In the present study, we assessed the relationship between ACSL4 mRNA expression and clinicopathological features. These results showed that ACSL4 mRNA expression was associated with gender, ccRCC subtypes, nodal invasion, tumor grade and cancer stages (P < 0.001, Fig. 3). To evaluate the association between ACSL4 mRNA expression and prognostic outcomes in ccRCC patients, Kaplan–Meier curves of OS and RFS were generated using the survival data from Kaplan-Meier Plotter (<https://kmplot.com/>). The results showed that the patients with high ACSL4 expression had significantly better OS (P = 0.014, Fig. 4A). Further subgroup analysis revealed that favorable OS in female patients (P = 0.013, Fig. 4C), patients with advanced tumor stage and grade (P = 0.13, P = 0.046, respectively, Fig. 4D-E), and patients with mutation burden (P = 0.013, Fig. 4F) was significantly correlated to high ACSL4 expression.

Overexpression of ACSL4 Promotes Ferroptosis

As we hypothesized that the dysregulation of ACSL4 may be contributed to ferroptosis in ccRCC, we first assayed the expression level of ACSL4 in 4 renal cancer cells. 786-O and 769-P cells expressed very low levels of ACSL4, whereas A498 and RCC4 expressed relatively high levels of ACSL4 (Fig. 5A). Then we determine whether ACSL4 expression regulates the anticancer activity of erastin (a ferroptosis inducer), ACSL4 cDNA was transfected into 786-O and 769-P cells. Overexpression of ACSL4 expression by gene transfection significantly aggravated the sensitivity to erastin-induced cell death in 786-O and 769-P cells (Fig. 5B-C), supporting that ACSL4 is a positive regulator of ferroptosis. Lipid peroxidation and Fe^{2+} accumulation are critical events in the induction of ferroptosis. We therefore assayed whether ACSL4 affects these events in ferroptosis. Indeed, overexpression of ACSL4 significantly increased erastin-induced lipid ROS production (Fig. 5D). In contrast, erastin-induced Fe^{2+} accumulation was not affected by overexpression of ACSL4 (Fig. 5E). These findings suggest that ACSL4 is a contributor of erastin induced ferroptosis by modulation of lipid peroxidation, but not iron accumulation.

Knockdown of ACSL4 Inhibits Ferroptosis

To further confirm the role of ACSL4 in ferroptosis, specific shRNA targeting ACSL4 was transfected into A498 and RCC4. Suppression of ACSL4 expression by RNAi remarkably inhibited erastin-induced cell death (Fig. 6A-B), suggesting that ACSL4 is a positive regulator of ferroptosis. Furthermore, we assayed the level of lipid peroxidation and iron accumulation in A498 and RCC4 cells after suppression of ACSL4 expression by shRNA. Lipid ROS level was decreased (Fig. 6C), but not Fe^{2+} accumulation (Fig. 6D) in A498 and RCC4 cells following erastin treatment. These findings further demonstrated that ACSL4 contributes to ferroptosis through increasing erastin-induced lipid peroxidation.

GO Functional Annotation and Pathway Enrichment of ACSL4

TCGA-KIRC Gene co-expression network analysis was performed to explore the underlying mechanism of ACSL4 participating in the malignant biological behavior of KIRC. We used the cBioPortal of Cancer Genomics to identify genes that were significantly coexpressed with ACSL4 in TCGA-KIRC. Results showed that 295 genes co-expressed with ACSL4 were identified by setting the absolute Spearman's $r \geq 0.5$ as a threshold. Then, these genes were imported into DAVID (<https://david.ncifcrf.gov/>) for further GO analysis and KEGG pathway analysis. The top 10 significant terms of GO functional annotation and KEGG pathway enrichment analysis were listed as Fig. 7. The most important pathway for gene enrichment of ACSL4 co-expression was Ubiquitin mediated proteolysis pathway (Fig. 7A). In the biological process and molecular function ontology, protein ubiquitination (GO: 0016567) and ubiquitin-protein transferase activity (GO: 0004842) constituted the majority of enriched GO categories (Fig. 7B-C). In the cellular component ontology, endocytic vesicle (GO: 0030139) ranked the top in the pathophysiological process (Fig. 7D).

Discussion

Ferroptosis was first described in 2012 when Dixon et al performed research on a small molecule termed erastin [7]. Ferroptosis is a new form of cell death dependent on iron and is associated with oxidative damage [9]. Features of ferroptosis include cytoplasmic and lipidic ROS accumulation, a reduction in mitochondrial volume, an increase in mitochondrial membrane density, rupture or loss of mitochondrial cristae and rupture of the mitochondrial outer membrane [10]. Recently, ACSL4 was demonstrated to serve as an essential component of ferroptosis through gene screening of Genome-Wide CRISPR and microarray analysis of anti-ferroptosis cell lines [11]. In the present study, the expression levels of ACSL4 and clinicopathological features in ccRCC were assessed. Additionally, markers of ferroptosis, including lipid ROS and Fe^{2+} was evaluated when ACSL4 was dysregulated. Therefore, it was concluded that ACSL4 is reduced in ccRCC and it was speculated that ferroptosis reduction may be associated with the pathogenesis of ccRCC.

It has been reported that ferroptosis is closely correlated with human diseases [12]. Studies on ferroptosis on ccRCC are limited. Heike and his colleagues suggested that reduced fatty acid metabolism due to inhibition of β -oxidation renders renal cancer cells highly dependent on the GSH/GPX pathway to prevent lipid peroxidation and ferroptotic cell death [13]. As a pivotal indicator and regulator of ferroptosis, ACSL4 functions as a critical determinant of ferroptosis sensitivity by modulating the cellular lipid composition. ACSL4-knockout cells were resistant to lipid peroxidation and ferroptosis [6]. ACSL4-mediated production of 5-hydroxyeicosatetraenoic acid (5-HETE) contributed to ferroptosis. Pharmacological inhibition of 5-HETE production by zileuton limited ACSL4 overexpression-induced ferroptosis [11]. ACSL4 enriches cellular membranes with long polyunsaturated ω 6 fatty acids. Moreover, ACSL4 is preferentially expressed in a panel of basal-like breast cancer cell lines and predicts their sensitivity to ferroptosis [11]. Ectopic expression of ACSL4 in ACSL4-negative prostate cancer (PCa) cells increases proliferation, migration and invasion, while ablation of ACSL4 in PCa cells expressing endogenous ACSL4 reduces cell proliferation, migration and invasion. The study further indicate that ACSL4 upregulates distinct pathway proteins including p-AKT, LSD1 and β -catenin [14]. As such, ACSL4 is regarded as a sensitive regulator of ferroptosis. However, ACSL4 has not been investigated in ccRCC associated with ferroptosis. In our study, we found that ACSL4 expression was down-regulated in tumor and related to poor prognosis, which suggest ACSL4 could serve as a biomarker and potential therapeutic target for ccRCC.

Similar to other cell death patterns, ferroptosis is closely associated with certain signaling pathways. Iron accumulation and lipid peroxidation are the key links associated with the occurrence of ferroptosis [10]. To validate the role of ACSL4 in ferroptosis in ccRCC, an ACSL4 overexpression plasmid or si-ACSL4 were transfected into four different ccRCC cell lines. The results indicated that the inhibition of ACSL4 can rescue cells from erastin-induced cell death and lipid ROS production. In contrast, erastin-induced Fe^{2+} accumulation was not affected by ACSL4 expression. As a fatty acid activating enzyme, the preferred substrates of ACSL4 are long-chain polyunsaturated fatty acids, such as AA and eicosapentaenoic acid. ACSL4 catalyzes these fatty acids and synthesizes the corresponding coenzyme A [10], which in turn affects the lipid peroxidation process.

In addition to the crosstalk between ferroptosis and other forms of cell death, the function and assay of ferroptosis related molecular and pathway need to be investigated. Some newly reported proteins such as PEBP1 [15], NCOA4 [16], and metallothionein-1G [17] are correlated with ferroptosis via iron metabolism and lipid peroxidation. In our GO and KEGG analysis, we found that the gene co-expressed with ACSL4 is enriched in the pathway of protein ubiquitination. BAP1 encodes a nuclear deubiquitinating enzyme to reduce histone 2A ubiquitination on chromatin. Recent studies reveal that BAP1 decreases H2Aub occupancy on the SLC7A11 (a ferroptosis inhibitor) promoter and represses SLC7A11 expression in a deubiquitinating-dependent manner and that BAP1 inhibits cystine uptake by repressing SLC7A11 expression, leading to elevated lipid peroxidation and ferroptosis. Furthermore, BAP1 inhibits tumour development partly through SLC7A11 and ferroptosis, and that cancer-associated BAP1 mutants lose their abilities to repress SLC7A11 and to promote ferroptosis [18]. This study reveals that, same as SLC7A11, ACSL4 ubiquitination may also be involved in the process of ferroptosis, which requires in-depth research.

Further studies on ferroptosis are needed not only to clarify the molecular mechanism but also to provide an opportunity for designing new therapeutic interventions. For example, in the treatment of advanced hepatocellular carcinoma, sorafenib resistance has been shown to result from the metallothionein-1G induced inhibition of ferroptosis [17]. Lachaier et al compared the levels of ferroptosis induced by sorafenib with those induced by the reference compound erastin in a panel of human cell lines originating from various cancer tissues. They found sorafenib induced ferroptosis in Caki-1 and ACHN (kidney tumors) cell lines. Also, they found a positive correlation between the ferroptotic potency of sorafenib and erastin. Compared to other kinase inhibitors, sorafenib is the only drug that displays ferroptotic efficacy, which establishes sorafenib as the first clinically-approved anticancer drug that can induce ferroptosis [19]. Doll and his colleagues further demonstrate that pharmacological targeting of ACSL4 with the antidiabetic compound class, thiazolidinediones, ameliorates tissue demise in a murine model of ferroptosis, suggesting that ACSL4 inhibition is a viable therapeutic approach to prevent ferroptosis-related diseases [11].

Conclusion

Our study demonstrated that ACSL4 is down-regulated in ccRCC and correlated with patients' clinicopathological features. ACSL4 suppresses proliferation of ccRCC cells, at least in part, by activating ferroptosis. These results indicate that ACSL4 may be regarded as a potential therapeutic target for the treatment of ccRCC and underlie future studies on pathways involving ACSL4 in a clinical setting.

Abbreviations

ACSL4

Acyl-CoA synthetase long-chain family member 4

RCC

renal cell carcinoma

ccRCC
Clear cell renal cell carcinoma
AA
arachidonic acid
TCGA
the Cancer Genome Atlas
DAVID
Database for Annotation, Visualization, and Integrated Discovery
qPCR
Quantitative real time polymerase chain reaction
PCa
prostate cancer
5-HETE
5-hydroxyeicosatetraenoic acid
CCK-8
Cell Counting Kit-8

Declarations

Ethics approval and consent to participate: This study was approved by the Academic Committee of Sun Yat-Sen University cancer center, and all patients provided informed consent.

Consent for publication: Written informed consent for publication was obtained from all participants.

Availability of data and materials: The data used during the study were provided by online datasets. Direct requests for these materials may be made to the provider as indicated in the “Material and methods”.

Competing interests: Not applicable

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Authors’ contributions: Na Guo conceived and designed the study. Na Guo performed the experiments and wrote the paper.

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Figures

Figure 1

Low expression levels of ACSL4 mRNA in ccRCC predicted by the Oncomine database. The Oncomine database mining analysis of ACSL4 mRNA levels in (A) Lenburg Renal [10], (B) Beroukhim Renal [11], (C) Gumz Renal [12], and (D) Jones Renal [13] grouped by ccRCC and normal kidney.

Figure 2

ACSL4 was significantly down-regulated in ccRCC tissues compared with normal kidney tissues. (A) ACSL4 mRNA expression was analyzed by qPCR in 16 pairs of ccRCC tissues and matched adjacent normal kidney tissues. ACSL4 IHC showed medium staining in normal kidney tissue (B); low staining in ccRCC tissue (C); not detected staining in ccRCC tissue (D). ***P < 0.001 vs normal

Figure 3

ACSL4 mRNA expression levels in patients with ccRCC in TCGA dataset cohorts. (A) Plot chart showing the expression of ACSL4 in ccRCC tissues compared with the normal controls. (B–F) Plots chart showing ACSL4 mRNA expression between male and female (B), KIRC (TCGA ccRCC dataset) subtypes (C), between the cases with or without nodal invasion (D), the cases with different tumor grade (E) and clinical pathological stages (F). ***P < 0.001 vs control

Figure 4

High ACSL4 expression was associated with favorable survival in patients with ccRCC. Kaplan–Meier curves of overall survival (OS) (A) and recurrence-free survival (RFS) (B) in all cases with primary ccRCC. Stratified analysis of OS according to the combination of ACSL4 and gender (C), tumor stage 4 (D), tumor grade 3 (E), and mutation burden (F), respectively.

Figure 5

Overexpression of ACSL4 promotes ferroptosis. (A) Western blot analysis of protein expression of ACSL4 in indicated cells. (B-C) Overexpression of ACSL4 promoted erastin-induced cell death in 786-O and 769-P cells ($n = 3$, *** $P < 0.001$ versus control cDNA group). (D-E) Lipid ROS and Fe²⁺ were assayed in 786-O and 769-P cells following treatment with erastin for 24 h ($n = 3$, ** $P < 0.01$ versus control cDNA group).

Figure 6

Knockdown of ACSL4 inhibits ferroptosis. (A-B) Knockdown of ACSL4 inhibited erastin-induced cell death in A498 and RCC4 cells ($n = 3$, $**P < 0.01$ versus control shRNA group). (C-D) Lipid ROS and Fe^{2+} were assayed in A498 and RCC4 cells following treatment with erastin for 24 h ($n = 3$, $**P < 0.01$ versus control shRNA group).

Figure 7

Gene ontology and pathway enrichment analysis of the ACSL4 co-expressed genes in TCGA-KIRC. The most important pathway for gene enrichment of ACSL4 co-expression was Ubiquitin mediated proteolysis pathway (A). In the biological process and molecular function ontology, protein ubiquitination (GO: 0016567) and ubiquitin-protein transferase activity (GO: 0004842) constituted the majority of enriched GO categories (B-C). In the cellular component ontology, endocytic vesicle (GO: 0030139) ranked the top in the pathophysiological process (D).