ORIGINAL ARTICLE

Lysosomal acid lipase promotes cholesterol ester metabolism and drives clear cell renal cell carcinoma progression

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Abstract

Objectives: Clear cell renal cell carcinoma (ccRCC) is characterized histologically by accumulation of cholesterol esters, cholesterol and other neutral lipids. Lysosomal acid lipase (LAL) is a critical enzyme involved in the cholesterol ester metabolism. Here, we sought to determine whether LAL could orchestrate metabolism of cholesterol esters in order to promote ccRCC progression.

Materials and methods: Quantitative reverse-transcription PCR and western blots were conducted to assess the expression of LAL in human ccRCC tissues. We analysed the relationship between LAL levels and patient survival using tissue microarrays. We used cell proliferation assays, colony formation assays, cell death assays, metabolic assays and xenograft tumour models to evaluate the biological function and underlying mechanisms.

Results: LAL was up-regulated in ccRCC tissue. Tissue microarray analysis revealed higher levels of LAL in advanced grades of ccRCC, and high LAL expression indicated lower patient survival. Suppressing LAL expression not only blocked the utilization of cholesterol esters but also impaired proliferation and cellular survival. Furthermore, immunohistochemistry staining showed that LAL expression was correlated with Akt phosphorylation. Suppressing LAL expression decreased the phosphorylation level of Akt and Src and reduced the level of 14,15-epoxyeicosatrienoic acids in ccRCC cells. Supplement of 14,15-epoxyeicosatrienoic acids rescued proliferation in vitro and in vivo.

Conclusions: LAL promoted cell proliferation and survival via metabolism of epoxyeicosatrienoic acids and activation of the Src/Akt pathway.

1 | INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the major pathological subtype of kidney cancer, with estimated 337 000 cases diagnosed and 143 000 deaths globally each year.¹ It is characterized histologically by accumulation of cholesterol, cholesterol esters (CEs) and other neutral lipids.²

Indeed, clear cell cancer tissue contains 8- and 35-fold higher levels of total cholesterol and esterified cholesterol, respectively, than found in normal kidney tissue.³ Hereditary clear cell renal cell carcinoma with t(3;8) translocation is frequently associated with disruption of the TRC8 gene, which encodes an E3-ubiquitin ligase for the cholesterol and fatty acid synthesis transcriptional regulators SREBP-1 and SREBP-2.⁴⁻⁶

Rather than having a passive role, several clues have indicated that dysregulated metabolism of cholesterol and CEs has an

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important function in various cancers.^{2,7-9} Solid tumours have access to circulating lipoproteins,^{7,10} and uptake and utilization of CEs are linked to cell proliferation, invasion and endoplasmic reticulum homeostasis.^{7,8,11-13} However, several studies have reported that high-grade ccRCC is associated with decreased lipid content.^{14,15} The mechanisms responsible for grade-dependent decreases in cellular lipids remain unknown, which is indicative of the complexity of aberrant lipid metabolism in ccRCC. Restructuring of lipid metabolism constitutes a recurrent pattern in ccRCC and correlates with tumour grade and prognosis.¹⁶

Lysosomal acid lipase (LAL) is a key regulator of CE metabolism. In lysosomes, LAL hydrolyses CEs and triglycerides (TGs) to produce free fatty acids (FFAs) and cholesterol.¹⁷ As the only hydrolyase that cleaves CEs in lysosomes, LAL is critical for degradation of CEs taken up by lipoprotein/scavenger receptors such as low density lipoprotein receptor (LDLR), lipoprotein receptor-related protein 1 (LRP1), very low density lipoprotein receptor (VLDLR) and CD36.¹⁸⁻²⁰ LAL also plays a vital role in recycling intrinsic lipids via autophagy (ie, lipophagy).²¹ The byproducts, free cholesterol (FC) and FFAs, can be utilized by cells, and any excess FC or FFAs are re-esterified and transported back to lipid droplets to serve as a reservoir. Thus, both uptake and recycling of CEs are regulated by LAL. LAL has been shown to affect the turnover of various bioactive lipids, such as retinoid,²² dehydroepiandrosterone²³ and oleoylethanolamide.²⁴

Furthermore, LAL regulates metabolism of long-chain fatty acids.²⁴⁻²⁶ It is known that compared to cholesterol oleate or saturated CEs, polyunsaturated CEs containing arachidonate are preferentially hydrolysed by LAL.²⁷ In addition, overexpression of LAL in *Caenorhabditis elegans* increases the level of arachidonic acid (AA).²⁴ Conversely, LAL deficiency leads to sequestration of arachidonate in CEs and TGs in the liver and spleen of rats.²⁸

Nonetheless, the role of LAL in cancer cells has yet to be clarified. Transcriptional analysis of data from The Cancer Genome Atlas (TCGA) revealed that LAL is up-regulated in ccRCC. Meanwhile, accumulating evidence has shown that lipoprotein receptor-lysosome pathways participate in cancer development and progression.^{7-9,13} LAL may either facilitate lipid uptake by digesting lipoprotein contents or mobilize intracellular CEs. Accordingly, we sought to determine whether LAL could play an important role in orchestrating CE metabolism to promote ccRCC progression. Here, we demonstrate LAL promoted cell proliferation and survival via metabolism of eicosanoid 14,15-epoxyeicosatrienoic acids (EET) and activation of the Src/Akt pathway in ccRCC.

2 | MATERIALS AND METHODS

2.1 | Samples

Tissue samples from 30 patients with ccRCC were obtained from Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine. This investigation was conducted in accordance with ethical standards and was approved by the authors' institutional review

board. This study conformed to Declaration of Helsinki. Informed consent was obtained from each patient.

2.2 | Cell culture

The human ccRCC cell lines 786-O, 769-P, Caki-1, OSRC-2 and human kidney tubule epithelial HK-2 cells were obtained from Type Culture Collection of the Chinese Academy of Sciences. All cell lines were authenticated and characterized by the supplier. The cells were expanded immediately and cryopreserved. Cells were used within 6 months of resuscitation. All cells were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, California, USA) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin and 100 g/mL streptomycin at 37° C in a humidified 5% CO₂ atmosphere.

2.3 | Immunohistochemistry (IHC)

Heat-induced antigen retrieval was applied using Tris/EDTA buffer (pH 9.0). Next, sections were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After a 30 minute FBS incubation to block non-specific protein binding, the primary anti-LAL antibody (Novus Biologicals, Littleton, Colorado, USA), anti-phospho-AKT (Cell Signaling Technology, Danvers, Maryland, USA), anti-AKT and anti-FOXO1 (Abcam, Baltimore, Maryland, USA) were applied followed by HRP-conjugated secondary antibody incubation (Santa Cruz Biotechnology, Dallas, Texas, USA). Diaminobenzidine (DAB) substrate solution was applied before haematoxylin counterstaining. Negative control assays were performed using normal IgG. All images were photographed using the Nikon Eclipse Ti microscope.

2.4 | Tissue Microarrays

Paraffin-embedded ccRCC tissue microarrays were obtained from US Biomax. The tissue microarray and IHC analyses were performed as previously described.^{29,30} The intensity of immunostaining was evaluated by two independent pathologists without knowledge of the clinicopathological data. A semi-quantitative H-score ranging from 0 to 300 was calculated by multiplying the staining intensity (0: negative, 1: weak, 2: moderate, 3: strong) by the distribution area (percentage of positive cancer cells, 0%-100%) at each intensity level for each sample.

2.5 | RNA extraction, cDNA synthesis and real-time PCR

RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions and reversely transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Bio Inc., Kusatsu, Shiga, Japan). For mRNA quantification, quantitative real-time RT-PCR (qRT-PCR) was performed using SYBR® green Premix Ex Taq™ kit (Takara Bio Inc., Japan)

according to the manufacturer's instructions. The forward and reverse primers set for human LAL were 5'-accagttatctctccacatac-3' and 5'-agtcaagatgctccattcc-3' respectively. The forward and reverse primers set for human 18s RNA (internal controls) were 5'-gtaaccgttgacccatt-3' and 5'-ccatcaatcggtagtagcg-3' respectively.

2.6 | siRNA transfection

Custom-designed siRNAs directed against LAL (siLAL1 GGCCAAAUUAGGACGAUATT, siLAL2 GGCAACAGCAGAGGA AAUATT), were synthesized and annealed (GenePharma, Shanghai, China). Cells were transfected with target and control siRNAs using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions.

2.7 | shRNA and cDNA transfection

The LAL (TRCN0000029245, TRCN0000029247) shRNA construct was obtained from TRC lentiviral shRNA Libraries (Broad Institute, Cambridge, Maryland, USA). The cDNA encoding human LAL was obtained by reverse transcription PCR and verified by sequencing. The LAL cDNA was then subcloned into the lentiviral vector pLVX-IRES-ZsGreen1 (Clontech, Mountain View, California, USA). Stably transfected cell lines expressing the cDNA and shRNA were generated as previously described.²⁹

2.8 | Western blot analysis

Cells were lysed using Radio Immunoprecipitation Assay (RIPA) buffer supplemented with protease and phosphatase inhibitors and protein concentration was measured using a BCA Protein Assay kit (Pierce, Waltham, Massachusetts, USA). Western blot analyses were as previously described.²⁹ The membranes were then incubated with following primary antibodies: anti-tubulin and anti-actin (Sigma, St. Louis, Missouri, USA), anti-LAL (Novus Biologicals, Littleton, Colorado, USA), anti-S6, anti-phospho-S6, anti-AKT and anti-phospho-AKT (Abcscitech, Baltimore, Maryland, USA), anti-Src, anti-p-Src, anti-ERK and anti-p-ERK (Cell Signaling Technology, Danvers, Maryland, USA). After incubation with primary antibody overnight at 4°C, HRP-conjugated secondary antibodies (Santa Cruz, Dallas, Texas, USA) were applied for 1 hour at room temperature. Target proteins were detected using an enhanced chemiluminescent solution (Millipore, Darmstadt, Germany).

2.9 | Cell proliferation

Cells were reversely transfected with siRNAs and plated in 6-well plates at a density of 4000 cells/well; this was considered day 1. Cell numbers were counted on days 2, 3 and 4. Alternatively, stably transfected cells were plated in 6-well plates at a density of 4000 cells/well, which was considered day 1. Cell numbers were then counted using the trypan blue exclusion method on days 2, 3 and 4.

For the bromodeoxyuridine (BrdU) analysis, cells were pulsed with 10 μ M BrdU (BD Biosciences, Sparks, Maryland, USA) for 5 hour, then trypsinized, fixed and labelled using BrdU Flow Kits (BD Biosciences, Sparks, Maryland, USA) according to the manufacturer's instructions, and analysed by flow cytometry using an FACSCalibur flow cytometer (BD Biosciences, Sparks, Maryland, USA).

2.10 | Plate colony formation assay

Two thousand cells were seeded and cultured for 2 weeks under indicated condition, then fixed for 30 minutes in 10% formaldehyde. Cell colonies were stained for 30 minutes with crystal violet (Sigma-Aldrich, St. Louis, Missouri, USA), washed and quantified.

2.11 | Cell death assay

Cells were seeded on 6-well culture plates. Each treatment group was seeded in triplicate. After overnight attachment, cells were treated with 8 nM paclitaxel. Following incubation, both attached and unattached cells were harvested. The percentage of cell death was measured using the trypan blue exclusion assay as previously described.³¹ For flow cytometry analysis, cells were labelled using Annexin V-FITC/PI kit (Yeasen, Shanghai, China) and analysed by flow cytometry according to the manufacturer's instructions.

2.12 | Metabolic assay

To measure intracellular arachidonic acid, OSRC-2 cells with shCtrl, shLAL1 and shLAL2 were washed with PBS and trypsinized. Arachidonic acid was extracted and analysed using ultra performance lipid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) by Metabo-profile Biotechnology Co., Ltd, Shanghai, China. The concentrations of arachidonic acid in samples were obtained by comparing to a set of standard samples of known concentration of arachidonic acid (ie, calibration curve). The data were normalized to cell counts.

To measure 14,15-EET/DHET, 80% confluent cells in 10 cm dishes were trypsinized and re-suspended in 2 mL of PBS with triphenylphosphine. 14,15 EET/DHET was extracted and measured using a 14,15-EET/DHET ELISA kit (Detroit R&D, Detroit, Michigan, USA) according to the manufacturer's instructions.

To measure total cholesterol and free cholesterol, 80% confluent cells in 6 cm dishes were trypsinized and re-suspended in a chloroform/isopropanol/Nonidet P-40 mix with a ratio of 7:11:0.1 respectively. Total cholesterol and free cholesterol were extracted and assessed using a cholesterol quantitation kit (Sigma, St. Louis, Missouri, USA).

2.13 | RNAseq data analysis

Raw files of the GSE46340 data set were downloaded from GEO³² websites. The Illumina universal adapter was trimmed using trim_galore. Reads were aligned to the mouse GRCm38 reference genome

using HISAT (2.0.1)³³ and counted using featureCounts (1.5.0).³⁴ We performed all subsequent analyses using limma³⁵ and R version 3.2.3 (<http://www.r-project.org/>).

2.14 | Network analysis

We used a slightly modified network analysis method described by Akbani³⁶ and Yasin et al.³⁷ The models were estimated using ARACNE-A³⁸ and Meinshausen-Buhlmann graph estimation.³⁹ Stability Approach to Regularization Selection (StARS) was applied for high-dimensional inference of undirected graphs.⁴⁰

2.15 | Quantification and correlation of IHC staining

Correlation of p-Akt and LAL staining were analysed using QuPath version 0.1.2 (<https://github.com/qupath/qupath/>). Briefly, tiles with 53*53 pixels were created on aligned images. Median optical density values of DAB (colour deconvolved) in each matched tiles were obtained and correlated.

2.16 | Animal experiments

All animal experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee. Female BALB/c nude mice were ordered from the Shanghai SLAC Laboratory Animal Co. Ltd. Shanghai, China. and subjected to subcutaneous tumourigenesis as described previously.²⁹ Briefly, 1×10^7 indicated cells resuspended in 200 μ L PBS were subcutaneously injected into the flanks of nude mice. Four weeks after injection, tumours were excised, weighed and assayed for protein expression. For the rescue experiment, 1 week following tumour cell inoculation, paratumoural injections were performed every 3 days for 15 days with 50 μ L EETs in coin oil (25 μ M) as previously described.⁴¹

2.17 | Statistical analysis

Statistical analysis was performed using R version 3.2.3 (<http://www.r-project.org/>). Experiments were performed at least three times independently. *P* values were 2-sided and *P* < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | LAL is up-regulated in human ccRCC and predicts survival

To initially gain insight into the expression pattern of LAL, we analysed public RNA-sequencing data from TCGA and found that LAL was up-regulated in ccRCC. Among all cancer types in TCGA programme, the ccRCC (kidney renal clear cell carcinoma, KIRC) cohort⁴² had the highest mRNA level of LAL (Figure 1A) indicating that LAL may play an important role in ccRCC. To validate the up-regulation of LAL at the transcriptional level in ccRCC, we compared LAL mRNA

levels in patient-matched ccRCC/normal tissues (*n* = 19 pairs) using qRT-PCR and found that LAL was significantly up-regulated in the former tissue (Figure 1B). We also compared LAL protein levels in patient-matched ccRCC/normal tissues (*n* = 10 pairs) using western blotting (Figure 1C). The protein level of LAL was higher in ccRCC samples compared with matched normal kidney tissue from the same patients (Figure 1C). The level of LAL in cancer and normal kidney tissues were quantified, and their ratios were represented as tumour to normal (T/N) ratios. Interestingly, when we compared the T/N ratios using the Fuhrman grading system, we found significantly higher T/N ratios for cancer with a higher grade (grades 3 and 4) compared with lower grades (grades 1 and 2) (Figure 1D). Furthermore, IHC showed LAL to be abundantly expressed in ccRCC tissues, whereas LAL expression was weaker in matched normal kidney tissue from the same patients (Figure 1E). Consistent with the results of western blotting, LAL expression was elevated in high-grade ccRCC. Then we evaluated the prognostic value of LAL using a public clinical tissue microarray of ccRCC samples collected from 90 patients between 2006 and 2008. LAL expression was evaluated by employing the widely applied H score (see the methods section for more details). We found LAL expression increased gradually and significantly in normal, grade 1-3 ccRCC tissues (Figure 1F).

Because the level of LAL expression was correlated with tumour grade, we inferred that up-regulation of LAL may promote ccRCC progression and predict poor prognosis. LAL expression groups were obtained using the median H score as the cut-off point. Log-rank test and Kaplan-Meier plot demonstrated significantly shorter overall survival time for patients with high tumour expression of LAL compared to the low-expression group (*P* < .05, Figure 1G).

3.2 | LAL is required for cell proliferation in kidney cancer cell lines

The level of LAL protein was higher in four ccRCC cell lines; 786-O, 769-P, Caki-1 and OSRC-2 cells, than in a normal kidney cell line, HK2 (Figure 2A). To further investigate the role of LAL, RNA interference (RNAi) was used to reduce LAL levels in human kidney cancer cell lines. After transient transfection of Caki-1 and 769-P cells with two independent LAL siRNAs (siLAL1 and siLAL2), decreased LAL levels were observed compared to the non-target control group (siCtrl) (Figure S1A, B). We next assessed the effect of LAL knockdown on kidney cancer cell proliferation by counting the number of Caki-1 and 769-P cells transiently transfected with siCtrl, siLAL1 and siLAL2 on days 1, 2, 3 and 4. Comparison with siCtrl-transfected cells revealed impaired proliferation capacity for siLAL1- and siLAL2-transfected cells (Figure S1C, D).

To investigate the long-term effect of LAL, we established Caki-1 and OSRC-2 cells stably infected with a non-target control shRNA (shCtrl) or two independent shRNAs targeting LAL (shLAL1 and shLAL2). The results showed a significant decline in LAL protein levels with shLAL1 and shLAL2 compared to control cells (Figure 2B, Figure S1E).

The Caki-1 and OSRC-2 cell proliferation rate were significantly attenuated following knockdown of LAL expression (Figure 2C, Figure S1F).

FIGURE 1 LAL is up-regulated in human ccRCC and predicts survival. (A), Transcription profile of LAL of various cancers included in TCGA programme (full names for TCGA cohort abbreviations were listed in File S1). The ccRCC (KIRC) cohort is emphasized using grey boxplot. (B), Real-time PCR analysis for LAL mRNA levels in human ccRCC tissue samples (T) and normal kidney tissues (N) (n = 19). 18S ribosomal RNA serves as an internal control. (C), Western blot of protein extracts from human ccRCC tissue samples (T) and normal kidney tissues (N) (n = 10). Levels of LAL in ccRCC tissue and normal kidney tissues were quantified and normalized using a gel pro analyzer. The ratio of LAL levels in ccRCC and normal kidney tissues are represented as the T/N ratio. (D), LAL T/N ratio in low-grade and high-grade ccRCC. (E), IHC analysis of low-grade and high-grade ccRCC and normal kidney tissue samples using either normal serum (NC) or the anti-LAL antibody. Images were photographed with a $\times 20$ objective lens using the Nikon Eclipse Ti microscope. (F), Boxplots of the H score of adjacent/normal kidney tissue and various grades of ccRCC (n = 90). (G), Kaplan-Meier survival curves of ccRCC patients with low vs high expression of LAL (n = 90; $P < .01$). *indicates $P < .05$

FIGURE 2 LAL is required for cell proliferation in kidney cancer cell lines. (A), Western blot of protein extracts of the normal kidney epithelial cell line HK-2 and 4 kidney cancer cell lines, 786-O, 769-P, Caki-1, OSRC-2 cells. (B), Western blot of protein extracts from Caki-1 cells stably transfected with shCtrl or shLAL1 and shLAL2. (C), Cell growth curves of Caki-1 cells stably transfected with shCtrl, shLAL1 or shLAL2. (D), FACS analysis for BrdU incorporation and DNA content (7AAD) of Caki-1 cells stably transfected with shCtrl, shLAL1 or shLAL2 and their quantifications. (E), Plate colony formation assay of Caki-1 and OSRC-2 cells stably transfected with shCtrl, shLAL1 or shLAL2 and their quantifications. (F), Western blot of protein extracts from Caki-1 cells stably transfected with the Ctrl vector or LAL cDNA. (G), Cell growth curves of Caki-1 cells stably transfected with Ctrl or LAL. (H), Quantifications of FACS analysis for BrdU incorporation and DNA content (7AAD) of Caki-1 cells stably transfected with Ctrl vector or LAL cDNA. *indicates $P < .05$

The BrdU incorporation assay showed that reduced S and G2/M-phase percentage was observed in Caki-1 cells with shLAL1 or shLAL2, accompanied by G1 phase arrest (Figure 2D). Similarly, the clonogenicity was also significantly attenuated following knockdown of LAL expression

(Figure 2E). However, Annexin V staining suggested that LAL suppression did not lead to a significant increase in apoptosis (data not shown).

To determine the effects of stable LAL overexpression on the proliferative activities of ccRCC cells, we established Caki-1 and OSRC-2

cells stably expressing either control or LAL cDNA (Figure 2F, Figure S1G) and counted cells on days 1, 2, 3 and 4 after plating in equal numbers. Proliferation was mildly increased in both cell lines stably expressing LAL cDNA compared to control cells (Figure 2G, Figure S1H). The BrdU incorporation assay showed that increased S-phase percentage was observed in Caki-1 cells with stably expressing LAL (Figure 2H).

3.3 | LAL is required for cholesterol ester metabolism in ccRCC

Since ccRCC up-regulates expression of several lipoprotein receptors^{18,20,43} and exhibits aberrant lipid metabolism, we investigated whether LAL plays an important role for CE metabolism in ccRCC. Thus, we detected cellular free cholesterol and CEs in OSRC-2 cells with shCtrl, shLAL1, shLAL2, control vector and an LAL expression plasmid. Similar to previous studies, knockdown of LAL resulted in an increase in CEs (Figure 3A). Free cholesterol was mildly decreased in shLAL-transfected cells compared with control cells (Figure 3B). Overexpression of LAL led to opposite results (Figure 3A-C), which is consistent with our finding of a grade-dependent increase in LAL and decrease in CEs. However, knockdown of LAL did not significantly alter the level of total free fatty acids (Figure 3C). Furthermore, we quantified CEs and FC in human ccRCC tissues of different Fuhrman grades ($n = 30$). Levels of CEs were significantly

decreased in higher-grade ccRCC tissue (grades 3 and 4) compared with lower-grade tissue (grades 1 and 2) (Figure 3D). Levels of FC were comparable or even slightly increased in higher-grade ccRCC. These alterations of CE and FC levels are consistent with the expression pattern of LAL. Taken together, these data show that LAL is required for CE metabolism in ccRCC.

3.4 | Suppression of LAL attenuates ccRCC growth and survival by regulating Akt phosphorylation

Although it has been reported that FoxO1 positively regulates LAL expression in adipocytes,⁴⁴ FoxO1 is negatively regulated by Akt and down-regulated in the majority of human renal tumour samples.^{45,46} Thus, we applied IHC to compare LAL, FoxO1 and phosphorylated Akt (p-Akt) (Ser473) and total Akt protein levels in adjacent slices of kidney and ccRCC samples. Interestingly, the level of p-Akt exhibited a negative correlation with FoxO1 (Figure 4A) and a strong positive correlation with LAL (Figure 4A, B) ($P < .05$). To determine the effects of FoxO1 on LAL expression in ccRCC cells, we induced FoxO1 activation via nutrient restriction and metformin according to previously described methods⁴⁴ in Caki-1 and OSRC-2 cells and found that activation of FoxO1 did not alter LAL mRNA expression (data not shown). In partial support of this, we found that

FIGURE 3 LAL is required for cholesterol ester metabolism in ccRCC. (A-C), Cholesterol esters (A), free cholesterol (B) and free fatty acid (C) contents of OSRC-2 cells with shCtrl, shLAL1, shLAL2, Ctrl and LAL. (D), Cholesterol esters (CEs), free cholesterol (FC) of low-grade (grades 1 and 2) and high-grade (grades 3 and 4) ccRCC tissue. *indicates $P < .05$

expression levels of LAL were similar in the kidney cancer cell lines RCC4 and UMRC2, with ectopic expression of FoxO1 and control vectors in a Gene Expression Omnibus (GEO) dataset (accession no. GSE23926).⁴⁶ Collectively, this evidence supports the hypotheses that FoxO1 does not alter LAL mRNA expression in ccRCC cells and that the effect of FoxO1 on LAL may be cell-type specific. Similarly, our manipulation of Akt activity using an activator and suppressor did not alter expression of LAL.

To investigate the relationship between LAL and Akt, we used western blotting to detect p-Akt, total Akt and downstream S6 in

Caki-1 cells with shCtrl, shLAL1 and shLAL2. Compared to shCtrl cells, Caki-1 cells carrying shLAL1 or shLAL2 displayed lower levels of Akt phosphorylated at Ser-374 (a surrogate of PI3K and Akt activities) and S6 phosphorylated at Ser-256 (a surrogate of mTOR activity) (Figure 5A). Taken together, these results indicate that LAL promotes Akt phosphorylation in ccRCC.

Moreover, we applied Akt activator sc-79 (the only Akt activator available) to restore the level of p-Akt in the shLAL1 group (Figure 5B). This Akt activator partially rescued the proliferation and colony defects induced by LAL suppression in Caki-1 and OSRC-2

FIGURE 4 Expression of LAL is correlated with expression of p-Akt in kidney and ccRCC tissues. (A and B), Expression of LAL, p-Akt, Akt and FoxO1 in normal kidney (A), and ccRCC (B), tissues, as determined by IHC. These regions with correlated expression of LAL, p-Akt and FoxO1 were highlighted with green dot lines. The upper row and lower row of p-Akt and LAL staining of ccRCC tissue (B) were quantified. The correlation plots represented median DAB intensities of p-Akt and LAL in matched small tiles. Normal kidney and ccRCC tissue slices were photographed using the Nikon Eclipse Ti microscope with a $\times 10$ and $\times 4$ objective lens

FIGURE 5 Suppression of LAL attenuates ccRCC cell growth and survival by regulating Akt phosphorylation. (A), Western blot of protein extracts from Caki-1 cells with shCtrl, shLAL1 or shLAL2. (B), Western blot of protein extracts from OSRC-2 cells with shCtrl, shLAL1 at 24 h after treatment with DMSO or sc79 (2 μ g/mL). (C, D), Cell growth curves of Caki-1 (C) and OSRC-2 (D) cells with shCtrl, shLAL1 and treated with DMSO or sc79. E, Plate colony formation assay of Caki-1 and OSRC-2 cells with shCtrl or shLAL1 and treated with DMSO or sc79. (F), Annexin V-FITC/PI assays and their quantification of paclitaxel-treated Caki-1 cells with shCtrl, shLAL1 or shLAL2. (G), Annexin V-FITC/PI assays and their quantification of paclitaxel-treated Caki-1 cells with Ctrl or LAL1. *indicates $P < .05$

FIGURE 6 LAL induces activation of c-Src. (A), Scatter plot of log₂ read counts of LAL mRNA and p-Src (Y416). (B), Network plot of LAL and signalling molecules from RNAseq and reverse-phase protein array data from TCGA. (C), Western blot of protein extracts from Caki-1 cells with shCtrl, shLAL1 or shLAL2. (D), Western blot of protein extracts from OSRC-2 cells with Ctrl or LAL

cells (Figure 5C-E), indicating that decreased p-Akt was required for suppressing proliferation in LAL knockdown cells.

Akt is a key regulator of both cell growth and survival, and previous research has shown that hyperactivated Akt can lead to pro-survival signals in ccRCC.⁴⁷ We therefore evaluated whether LAL-dependent activation of Akt contributes to the survival of ccRCC cells. Several studies have utilized various means to efficiently enhance the sensitivity of ccRCC cells to the chemotherapy drug paclitaxel.⁴⁷⁻⁵⁰ We performed Annexin V/PI assays and cell viability assays (trypan blue exclusion test) using paclitaxel-treated Caki-1 and OSRC-2 cells with shCtrl, shLAL1 and shLAL2. Knockdown of LAL raised the percentage of cell death (Figure 5F, Figure S2A, B). In contrast, LAL overexpression partially blocked cell death in these cells (Figure 5G, Figure S2C, D).

3.5 | LAL induces activation of Src

To further elucidate the mechanism of decreased Akt phosphorylation in cells knocked down for LAL, we merged TCGA reverse-phase protein array data for ccRCC samples with normalized RNAseq log₂ counts of LAL. Pairwise correlation tests revealed that the link between phosphorylation (Y416) of c-Src (which activates c-Src) and LAL outperformed all other pairs (correlation coefficient = 0.31; Benjamini-Hochberg adjusted *P* value = 7.36E-10) (Figure 6A). Furthermore, to better understand the regulatory structure, we performed unbiased data-driven signalling network analysis without the inclusion of prior knowledge using Meinshausen-Buhlmann Graphical estimation³⁹ and mutual information-based Algorithm for the Reconstruction of Accurate

Cellular Networks (ARACNE-A).³⁸ A representative ARACNE-A model is presented in Figure 6B. LAL was stably linked with p-Src in both models, in which the shortest path between LAL and p-Akt was LAL/p-Src (Y416)/p-ERK1/2 (T202/Y204)/p-Akt (S473). It is well known that Src is capable of activating both ERK1/2 and Akt depending on the circumstances.

To determine whether Src is upstream of both LAL and Akt, we compared transcriptional profiles of mouse embryonic fibroblasts stably transfected with a retroviral vector/constitutively active mutant Src (Y527F) and the livers of wild-type/Src transgenic mice using public datasets GSE46340 and GSE15815. In both data sets, activated Src did not alter the mRNA level of LAL (Figure S3A, B). Therefore, we speculated that LAL-mediated Akt activation is dependent on Src.

To test whether Src contributes to LAL-mediated Akt activation, we compared the level of p-Src (Y416) in OSRC-2 cells harbouring shCtrl, shLAL1 and shLAL2. Suppression of LAL decreased the level of p-Src in these cells (Figure 6C). Interestingly, the basal levels of p-ERK and p-STAT3 were unchanged after suppression of LAL. In contrast, overexpression of LAL led to higher levels of p-Src and p-Akt (Figure 6D).

3.6 | LAL promotes phosphorylation of Src by regulating metabolism of epoxyeicosatrienoic acids

Although LAL did not alter total free fatty acid levels, we noticed that LAL changed the intracellular concentration of long-chain fatty acids such as arachidonic acid (AA).^{24,28,51} Given defects in the intracellular transformation of linoleic acid to AA in ccRCC,⁵² we speculate

FIGURE 7 LAL promotes phosphorylation of Src by regulating metabolism of epoxyeicosatrienoic acids. (A), Growth fold changes of OSRC-2 cells treated with the indicated concentration of arachidonic acid. (B), qRT-PCR analysis of CYP2J2 mRNA levels in human ccRCC tissue samples (T) and normal kidney tissues (N) ($n = 14$). 18S ribosomal RNA serves as an internal control. (C), 14,15-EET ELISA assay of extracts of Caki-1 cells with shCtrl, shLAL1 and shLAL2. (D), Western blot of protein extracts from OSRC-2 cells with shCtrl or shLAL1 and treated with the indicated concentrations of 14,15-EET for 45 min. (E), 14,15-EET ELISA of extracts of OSRC-2 cells with shCtrl or shLAL1 and treated with normal foetal bovine serum or lipoprotein-deficient serum. (F, G), Cell growth curves of Caki-1 (F) and OSRC-2 (G) cells with shCtrl or shLAL1 and treated with DMSO or 14,15-EET. H, FACS analysis for BrdU incorporation and DNA content (7AAD) of Caki-1 cells stably transfected with shCtrl or shLAL1 and treated with DMSO or 14,15-EET. I, Percentage of cell death for paclitaxel-treated Caki-1 (I) cells with shCtrl or shLAL1 and treated with DMSO or 14,15-EET according to annexin V and propidium iodide (PI) staining. *indicates $P < .05$

that LAL modulates the c-Src/Akt pathway through the AA-derived signal eicosanoid. However, the effects of AA on cancer cells are controversial. Accordingly, we treated Caki-1 and OSRC-2 cells with

a wide range of AA concentrations and evaluated cell proliferation. Interestingly, the minimum cytotoxic concentration varied considerably between these ccRCC cells. The commonly reported treatment

TABLE 1 Change in intracellular arachidonic acid after LAL knockdown in OSRC-2 cells

Name	Concentration ($\mu\text{mol}/10^7$ cells)
OSRC-2 shCtrl	0.40 ± 0.09
OSRC-2 shLAL1	0.14 ± 0.02^a
OSRC-2 shLAL2	0.14 ± 0.05^a

^aSignificant difference with respect to control group (< 0.05); $n = 3$.

concentration of 50 μM was capable of killing Caki-1 and OSRC-2 cells, whereas a low concentration of AA induced the cells to proliferate (Figure 7A). In addition, the concentration with the peak stimulating effect was much lower than that (50 μM) commonly reported in the literature. Unbiased metabolomics revealed that AA is increased in high-grade ccRCC,¹⁹ which is consistent with our finding of a grade-dependent increase in LAL. Then, we confirmed that LAL regulates the level of AA (Table 1) using mass spectrometry. These data collectively imply that c-Src/Akt activation may be linked to a pathway that is dependent on AA metabolism.

Furthermore, we evaluated expression of all enzymes that metabolize AA using a public microarray dataset of normal kidney and ccRCC (GSE40435)⁵³ (Figure S4A) and found that the cytochrome P450 CYP2J2 was drastically up-regulated in ccRCC tissue. This was also confirmed using qRT-PCR (Figure 7B). We then notice that 14,15-EET, the most abundant EET regioisomer generated by CYP2J2, acts as a potent mitogen in renal epithelial cells, leading to activation of Akt in a Src-dependent manner.⁵⁴⁻⁵⁷ In addition, 14,15-dihETrE, a downstream metabolite of 14,15-EET, was significantly elevated in high-grade ccRCC compared with low-grade carcinoma.¹⁹

To explore the possible role of LAL in 14,15-EET production, the cellular 14,15-EET content was measured in shCtrl and shLAL Caki-1 cells, with a significantly lower level found in the latter (Figure 7C). Furthermore, 14,15-EET supplementation activated both p-Src and p-Akt in OSRC-2 cells (Figure 7D, quantification of p-Src and p-Akt: Figure S4B, C). To investigate the role of lipoprotein lipids on 14,15-EET, we treated shCtrl/shLAL1 OSRC-2 cells with foetal bovine serum (FBS) or lipoprotein-deficient serum and found that the latter lowered 14,15-EET levels in both shCtrl and shLAL1 OSRC-2 cells (Figure 7E).

The growth kinetics of Caki-1 and OSRC-2 cells with different levels of LAL and 14,15-EET treatment were compared by counting cell numbers on days 1, 2, 3 and 4 after plating equal numbers of cells. After adherence, medium containing 14,15-EET was refreshed

every 4-6 hours. 14,15-EET treatment of both cell lines with shLAL1 partially restored proliferation (Figure 7F-G) and the BrdU incorporation assay showed that 14,15-EET treatment partially restored S-phase percentage in Caki-1 cells with stably expressing LAL (Figure 7H). Consistent with previous results, 14,15-EET treatment of shLAL1 Caki-1 and OSRC-2 cells partially blocked paclitaxel's cytotoxic effects (Figure 7I, Figure S4D, E).

3.7 | LAL suppression inhibits tumour growth in vivo

To examine the effects of LAL knockdown on the in vivo tumorigenicity of kidney cancer cells, Caki-1 cells stably transfected with shCtrl or shRNAs targeting LAL (shLAL1) were injected subcutaneously into nude mice. Tumour growth significantly reduced in the shLAL1 group compared with the shCtrl group (Figure 8A-C). Moreover, western blot analysis of the excised tumours confirmed the maintenance of both the LAL knockdown and decreased Src/AKT/mTOR activity phenotypes (Figure 8D). Moreover, paratumoural injected with 14,15-EET partially reversed shLAL1's impact on tumour growth (Figure 8E, F).

4 | DISCUSSION

Rather than increasing de novo synthesis of fatty acids and cholesterol, ccRCC up-regulates expression of several lipoprotein receptors such as LRP1, VLDLR and CD36 in order to absorb extracellular lipids.¹⁸⁻²⁰ However, the importance of extracellular lipid utilization remains relatively poorly defined. In the present study, we first established that LAL was overexpressed in human ccRCC tissues. We herein show that LAL expression was positively correlated with histological grade and that ccRCC patients with high LAL expression exhibited a poor prognosis. More intriguingly, LAL was required for CE metabolism in ccRCC cells (Figure 3). LAL depletion led to accumulation of CEs. Furthermore, we revealed a novel function for LAL in regulating 14,15-EET metabolism and the activities of Src/Akt, in order to support the proliferation and survival of ccRCC cells (Figure 8G).

Our study, along with previous findings, shows that LAL depletion decreases proliferation of ccRCC cells (Figure 2) and bone marrow mesenchymal stem cells (MSCs).⁵⁸ Furthermore, most evidence indicates the depletion of upstream LDL receptors and scavenger receptors, leading to decreased proliferation in many solid tumours, such as breast and pancreatic cancers.^{7,9} However, it has

FIGURE 8 LAL suppression inhibits tumour growth in vivo. (A), Picture of subcutaneous tumours from nude mice subcutaneously injected with Caki-1 cells stably with shCtrl or shLAL1. (B, C), Weight (B) and size (C) of subcutaneous tumours from nude mice subcutaneously injected with Caki-1 cells stably with shCtrl or shLAL1 ($n = 9$). (D), Western blot of protein extracts from subcutaneous tumours from nude mice subcutaneously injected with Caki-1 cells stably with shCtrl or shLAL1 at 30 days after injection. (E, F), Weight (E) and size (F) of subcutaneous tumours from nude mice subcutaneously injected with Caki-1 cells stably transfected with shCtrl or shLAL1 and paratumoural injected with 14,15-EET ($n = 10$). *indicates $P < 0.05$. (G), A, schematic working model for the functions of LAL in ccRCC. LAL is required for CE metabolism in ccRCC cells; LAL promotes cell proliferation and survival via metabolism of epoxyeicosatrienoic acids and activation of the Src/Akt pathway

been reported that LAL depletion stimulates myeloid-derived suppressor cell (MDSC) growth.⁵⁹ Context-dependent mechanisms may be involved. LAL has also been proved to regulate M2 macrophage activation,⁶⁰ surfactant homeostasis in the lung⁶¹ and T-cell development.⁶² An in-depth analysis is required to confirm the differential roles of LAL in different types of cells.

Previous studies have shown that LAL participates in lipoprotein receptors/lysosome pathways and lipophagy-mediated cholesterol efflux.²¹ Thus, both uptake and degradation of CEs are facilitated by LAL. Moreover, LAL modulates CE dynamics by cooperating with up-regulated lipoprotein receptors (LRP1, VLDLR and CD36) and cholesterol efflux-related genes (ABCA1 and ABCG1). In the present study, we found that LAL decreased the level of CEs and increased the level of FC (Figure 3), which is consistent with trends of CEs and FC in different grades of ccRCC tissues. Interestingly, fold-changes of FC and FFA are significantly smaller than those of CEs. In fact, unlike CEs, intracellular levels of FC and FFAs are tightly regulated. Excessive FC will be re-esterified or exported through reverse cholesterol transport, while depletion of FC will activate its de novo synthesis process. Similarly, excessive FFAs will be consumed during the formation process of CEs and triacylglyceride, while depletion of non-essential FFAs will also activate their de novo synthesis processes. The composition of FFAs is often changed following LAL alteration.²⁴ It has been reported that LAL preferentially hydrolyses polyunsaturated cholesterol esters.²⁷ Depletion of LAL could stimulate de novo synthesis of non-essential fatty acids (data not shown). These may be the reasons through which LAL alters the level of AA rather than total FFAs. It is interesting to observe that LAL depletion reduces the 14,15-EET production in lipoprotein deficient (LPDS) medium (Figure 7). It is known that LAL recycles intrinsic lipids. One possible mechanism is that LAL can mobilize intrinsic lipids and release AA which can be converted into 14,15-EET.

Interestingly, we observed that LAL exhibited a strong positive correlation with p-Akt (Figure 4). In general, lysosome-related genes are negatively regulated by the Akt/mTOR pathway.⁶³⁻⁶⁵ Why lysosomal gene LAL been co-expressed with Akt remains an open question. Although constitutive activation of the PI3K/Akt/mTOR pathway in high-grade cancer usually suppresses autophagy-lysosome processes, LAL does not appear to exhibit this tendency in ccRCC. One interesting point is that LAL is highly homologous to human gastric lipase, which plays an important role in the digestion of dietary TGs in the gastrointestinal tract.⁶⁶ We presume that metabolic signals from LAL maybe evolutionarily interpreted as a plentiful nutrient supply, activating nutrient sensors and promoting cell growth.

We have found that LAL regulates the level of epoxyeicosatrienoic acids and the activity of Src and Akt in ccRCC cells (Figure 7C, D). It has been reported that epoxyeicosatrienoic acids exert their mitogenic effects predominantly through a Src kinase-mediated pathway.⁵⁵ However, certain previous studies have shown that EETs are capable of activating epidermal growth factor receptor (EGFR) in endothelial cells and cancer cells.^{54,67}

In addition, it has been reported that Src phosphorylates and activates EGFR⁶⁸ and that EGFR stimulation leads to activation of Src.⁶⁹ Nonetheless, additional work is required to address whether 14,15-EET-derived Src-Akt signalling required the involvement of EGFR in ccRCC.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to the work presented.

AUTHORS' CONTRIBUTIONS

JW and JQ designed the experiments and wrote the paper; JW, MT, JG, PZ, JZ, LT and QW performed the experiments; JW and MT summarized the data and performed the statistical analysis. XT provided the technical advice and material supports. All the authors have read and approved the final manuscript for publication.

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SUPPORTING INFORMATION

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