Int J Clin Exp Med 2019;12(4):3261-3271
www.ijcem.com /ISSN:1940-5901/IJCEM0077757

Original Article

The effects of LOXL2 in clear cell renal cell carcinoma: a preliminary study in the Chinese population

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Received April 11, 2018; Accepted December 11, 2018; Epub April 15, 2019; Published April 30, 2019

Abstract: Background: An abnormal expression of lysyl oxidase-like 2 (LOXL2) has been reported and is considered to play an important role in several cancers, such as breast and pancreatic cancer and esophageal squamous cell carcinoma. However, studies on the expression status and function of LOXL2 in clear cell renal cell carcinoma (ccRCC) remain limited, and their results are contradictory. Aim: To investigate the expression status of LOXL2 in Chinese patients with ccRCC and its functional significance. Methods: Using quantitative reverse transcription PCR, we compared the level of LOXL2 mRNA between ccRCC and corresponding adjacent non-cancerous tissues from 10 Chinese patients, and among 4 different ccRCC cell lines (786-O, A-498, ACHN, and Caki-1) and the normal renal cell line HEK293. The LOXL2 small interfering RNA (siRNA) lentivirus vector was constructed and used to infect 786-O cells to down-regulate LOXL2 expression. Subsequently, cell proliferation, colony formation, the cell cycle, and apoptosis were detected in these infected 786-O cells. Results: Significantly over-expressed LOXL2 was confirmed in the ccRCC specimen and 4 cancer cell lines compared to the normal control group (all P values < 0.05). LOXL2 down-regulation significantly inhibited 786-O cell proliferation and colony formation ability, induced cell cycle arrest in the G2/M phase and promoted cancer cell apoptosis. Conclusion: LOXL2 is highly expressed in Chinese patients with ccRCC and probably plays an important functional role in ccRCC tumorigenesis.

Keywords: Lysyl oxidase-like 2, kidney, carcinoma

Introduction

In China, about 66,800 people are newly diagnosed with kidney cancer, and 23,400 patients die of the disease each year [1]. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of kidney cancer, accounting for 75%-85% of kidney cancer cases [2-4]. Currently, surgical resection and targeted therapy are the main therapeutic approaches for ccRCC [5]. Novel molecule-targeted drugs and immunotherapy agents are under development, and some have been approved with significant activity in ccRCC patients [6, 7]. Despite these advances, a large number of patients still die of the disease. Developing novel biomarkers and alternative therapeutic targets is still valuable.

Lysyl oxidase-like 2 (LOXL2), a copper-dependent enzyme, is a member of the lysyl oxidase family which oxidizes primary amine substrates to reactive aldehydes [8, 9]. It catalyzes the covalent crosslinking of collagen and elastin in the extracellular matrix (ECM) and plays an important role in normal tissue development and homeostasis [10]. Aberrantly bidirectional expression of LOXL2 has been observed in several solid tumors and found to be related to tumorigenesis [11]. With the exception of down-regulation in a minority of cancers such as head and neck squamous cell carcinoma [12] and lung adenocarcinoma [13], the up-regulation of LOXL2 expression has been demonstrated in most tumors, including breast [14], colon [15], esophageal [15], pancreatic [16, 17] and gastric cancer [18]. Furthermore, it was reported that the inhibition of over-expressed LOXL2 using a specific antibody and inhibitor could effectively reduce the primary breast tumor burden [19], invasion and migration [20], and prohibit tumor growth in primary and metastat-

ic xenograft models of breast and ovarian cancer [21].

The expression status of LOXL2 in ccRCC is not completely known. So far, only a handful of studies have reported on the high expression of LOXL2 in clinical ccRCC specimens [22-24], and the number of relevant studies is considered limited. In addition, the effect of aberrant LOXL2 expression on ccRCC tumorigenesis remains unknown. Considering its bidirectional expression [11], its significant role in tumorigenesis, and its potential as a therapeutic target in other cancers, it is necessary to measure the expression level of LOXL2 in Chinese patients with ccRCC and explore its function in ccRCC cells by regulating its expression reversely.

Materials and methods

Patients and tissue samples

A total of 10 pairs of ccRCC specimens and corresponding adjacent non-cancerous tissues (ANTs) were obtained from ccRCC patients after radical nephrectomy at Sichuan University West China Hospital in China from June 2013 to August 2013. Clinicopathological data were obtained from archived medical records. The collected data included patient gender, age, body mass, hypertension history, side, tumor grade, and clinical and pathological stage. All ccRCCs were diagnosed by pathology. The histological grade was subdivided into three grades as suggested by Störkel [25]. Clinicopathological staging was determined according to the 2009 TNM classification system of the International Union Against Cancer (UICC) [26]. All patients were followed up every 3 to 6 months in the first 2 years and then once yearly. Prior written and informed consent was obtained from each patient. This study was approved by the ethics review board of Sichuan University West China Hospital.

Cell culture

Four human ccRCC cell lines containing 786-0, A-498, ACHN and Caki-1, and the normal renal cell line HEK293 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 786-0 cells were cultured in RPMI-1640 medium. A-498, ACHN and HEK 293 were cultured in an EMEM medium. Caki-1 cells were cultured in McCoy's 5A medium. 10% fetal bovine serum, 100 U/ml penicillin G, and 0.1 μg/ml streptomycin were supplemented. These cells were all cultured in the humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Production of an RNA interference gene with a lentiviral vector

Cells that were transfected with LOXL2-small interfering RNA (LOXL2-siRNA; 5'-ATT ACT CCA ACA TCAT-3') were used for LOXL2 inhibition. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA) was used. Cells that expressed scrambled short hairpin RNA (shRNA; 5'-TTC TCC GAA CGT GTC ACGT-3') in a lentiviral vector were used as the control. A human LOXL2 dsDNA oligonucleotide sequence was synthesized with the targeted siRNA sequence (GeneChem Co., Ltd., China). A pGCSIL-green fluorescent protein (GFP) plasmid (GeneChem Co., Ltd., China) was digested by Age I/EcoR I (GeneChem Co., Ltd., China) and connected with the dsDNA sequence, and then transformed into competent *E*. coli. Lentiviral vector construction and infection were performed as previously described [27]. Cell lines with stable LOXL2 shRNA expression were selected on lysogeny broth (LB) agar medium after 16 h culturing at 37°C. The cells were identified by PCR using a Taq polymerase kit (Takara Bio, Inc., Otsu, Japan). The sense and anti-sense primers were 5'-CCT ATT TCC CAT GAT TCC TTC ATA-3' and 5'-GTA ATA CGG TTA TCC ACG CG-3', respectively. PCR was performed at 94°C for 30 sec, followed by 30 cycles at 94°C, 55°C and 72°C for 30 sec, and 72°C for 6 min. The positive clones of recombinant plasmids were sequenced and extracted.

Quantitative RT-PCR

Total RNAs were extracted from clinical samples and cell lines using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., USA) and cDNA was obtained by reverse transcription using the Moloney-Murine Leukemia Virus Reverse Transcriptase cDNA Synthesis kit (Promega Corporation, USA), according to the manufacturer's protocols. Then the mRNA expression level of LOXL2 was measured by real-time PCR using the SYBR Premix Ex Taq kit (Takara Bio, Inc., Japan). The primer sequences were as follows: sense, 5'-GTC TGC GGC ATG TTT GG-3' and anti-sense, 5'-GCT CTG GCT TGT ACG CTTT-3' for LOXL2; and sense, 5'-TGA CTT CAA CAG CGA CAC CCA-3' and anti-sense, 5'-CAC CCT

GTT GCT GTA GCC AAA-3' for GAPDH (the internal control). Optimization of the PCR reaction was conducted according to the manufacturer's instructions. A melting curve analysis was conducted to check the amplification. The fold change of the target gene was normalized to GADPH. The data were calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

Western blot

The inhibitory effect by siRNA on the LOXL2 protein level in 786-0 cells was examined using Western blot. Infected cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. Lysates were collected after treatment. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Immunoblotting was performed with rabbit anti-LOXL2 antibodies (1:1000; ab96-233; Abcam, Cambridge, UK). Anti-GAPDH antibodies (1:1000; ab8245; Abcam, Cambridge, UK) were used as an internal loading control. The intensities of the protein bands were detected using an enhanced chemiluminescence system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Cell counts

O cells were infected with a lentiviral vector. The cells were seeded at a density of 5×10^4 cells/well and incubated in 6-well plates. A Cellomics™ instrument (ArrayScan VT1; Thermo Fisher Scientific, Inc., USA) was used to measure the infected cells, which were stained with fluorescent green. The numbers of the LOXL2-siRNA treated and control cells were investigated for five days. A cell growth curve was constructed after calculating and analyzing these parameters.

BrdU labeling assay

Infected cells with an initial density of 2×10^4/well were seeded in 96-well plates. A BrdU kit (Roche Diagnostics, Basel, Switzerland) was used and diluted in 1:1000 ratio. The cells were incubated with diluted BrdU solution (10 µl/well) and fixed. Subsequently, the cells were stained with anti-BrdU antibodies for 90 min at room temperature. The cells were examined at 24 and 96 h. The absorbance was measured at a wavelength of 450 nm and 630 nm using a microplate reader. All experiments were carried out in triplicate. The fold changes in cell proliferation were calculated, analyzed, and are presented in the figures.

Colony formation assay

In 6-well plates, the infected cells were seeded at a density of 1×10^3 cells/well and cultured for 14 days. The culture medium was replaced every 3 days. Cell colonies were observed and recorded using a fluorescence microscope (AX10 imager A2/AX10 cam HRC, Zeiss, Germany) on day 14. Then, the colonies were fixed with paraformaldehyde for 30-60 min before staining with Giemsa for 20 min. Then we washed the cells with ddH_2O until the plate background was clean and then we allowed it to air dry. The colonies were counted using a microscope (Micropublisher 3.3RTV; Olympus Corporation, Tokyo, Japan). All the experiments were conducted in triplicate and the data were analyzed using CellQuest Pro software (version 5.1.1).

Flow cytometry to detect cell cycle

The infected cells were harvested when they reached 80% confluence in a 6-well dish. The cells were fixed using 70% ethanol at 4°C for at least 1 h. Then the cells were centrifuged at 1500 rpm for 5 min at room temperature and the ethanol was decanted thoroughly. After being washed and collected, the fixed cells were treated with 10 µg/ml RNase A and propidium iodide (50 µg/ml) at room temperature. The cells were then resuspended. The cell cycle was analyzed using flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest Pro software (version 5.1.1). In the experiment, the DNA of these cells was stained by propidium iodide, which is a fluorescent molecule. The cells had more DNA in the S phase than in the G1 phase. They took up more dye proportionally and fluoresced more brightly until they had doubled their DNA content. Cells in the G2/M phase were approximately twice as bright as the cells in G1 phase. This experiment was repeated three times.

Flow cytometry to detect apoptosis

The cells were harvested and washed with D-Hanks (Haling Biotechnology Co., Ltd., Shanghai, China). Subsequently, they were centri-

Table 1. Clinicopathological data of ten ccRCC patients

Valuable	Parameter
Age (year)	53 ± 10.4 (range 45-60)
Gender (No.)	
Male	7
Female	3
Body mass (kg)	61.4 ± 16.0 (range 40-93.5)
Hypertension (No.)	
Yes	3
No	7
Side (No.)	
Left	6
Right	4
Pathological grade (No.)	
Well differentiated	3
Moderately differentiated	5
Poorly differentiated	2
pT stage (No.)	
T1a	2
T1b	8
cN stage (No.)	
N0	10
cM stage (No.)	
M0	10
TNM classification (No.)	
I	10

pT stage, pathologically primary tumor stage; cN stage, clinically regional lymph node stage; cM stage, clinically distant metastasis stage.

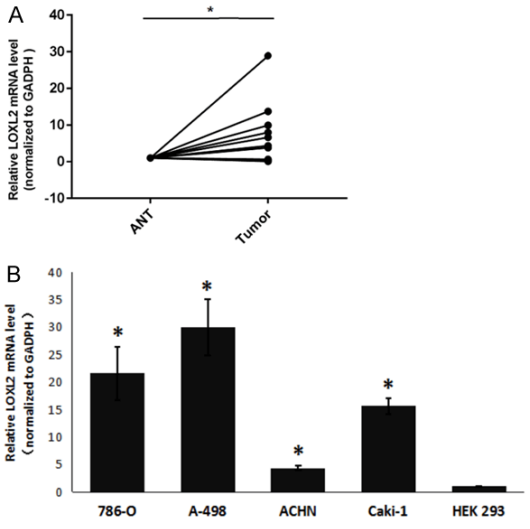

Figure 1. Relative LOXL2 mRNA levels normalized to GADPH in clinical specimens and cell lines. A. LOXL2 mRNA levels in clinical ccRCC tissues were significantly higher than those in ANT. B. LOXL2 mRNA levels in ccRCC cell lines (786-O, A-498, ACHN and Caki-1) were significantly higher than those in normal renal cells (HEK293). ANT, adjacent non-cancerous tissue; LOXL2, lysyl oxidase-like 2; ccRCC, clear cell renal cell carcinoma. *P < 0.05 vs. the control group.

fuged at 1500 rpm for 5 min at room temperature. The precipitated cells were washed once with PBS and centrifuged again. After being washed with a binding buffer, the collected cell suspension with a final density of 1×10^6-1×10^7 cells/ml was gathered and a 5 μl Annexin V Apoptosis Detection kit APC (eBioscience, Inc., San Diego, CA, USA) was added and stained for about 15 min at room temperature in a dark room. A flow cytometry analysis was then performed. This experiment was repeated three times.

Statistical analysis

All data were expressed as the mean ± standard deviation and the intergroup difference was compared using an independent samples *t*-test, except for comparing the LOXL2 mRNA levels between the tumor tissues and ANT, between which a paired t-test was performed. We used a chi-square test to compare the rates between groups. These analyses were conducted using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). A *P* value < 0.05 was considered statistically significant.

Results

Characteristics of the ccRCC patients

Ten ccRCC patients from China who underwent a radical nephrectomy were selected for this study. No regional lymph node or distant metastasis was found when ccRCC was first diagnosed. The characteristics of these patients are summarized in **Table 1**. Nine patients have been followed up for 4 years without cancer recurrence or metastasis. One patient had lung metastasis 9 months postoperatively and died about one and half years after surgery.

LOXL2 expression was up-regulated in clinical ccRCC specimens and cell lines

The mRNA level of LOXL2 was significantly higher in ccRCC tissue than it was in the corresponding ANT (*P* < 0.05; **Figure 1A**). In order

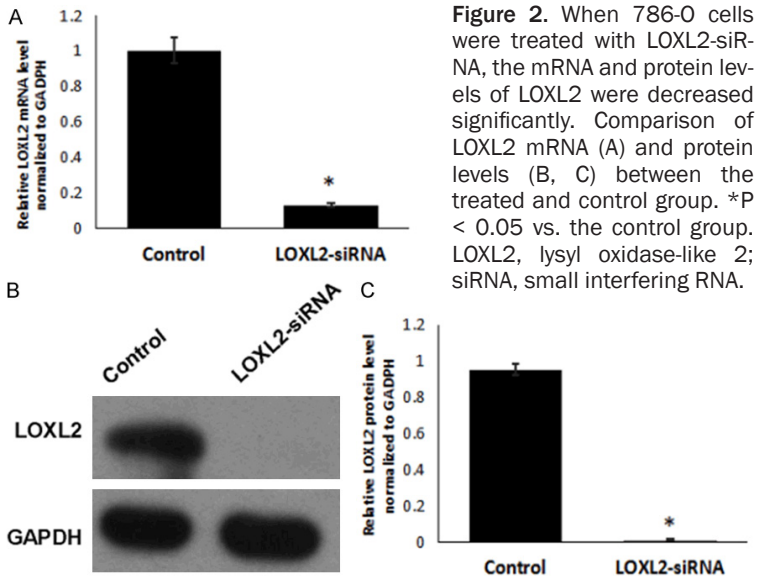

Figure 2. When 786-0 cells were treated with LOXL2-siRNA, the mRNA and protein levels of LOXL2 were decreased significantly. Comparison of LOXL2 mRNA (A) and protein levels (B, C) between the treated and control group. *P < 0.05 vs. the control group. LOXL2, lysyl oxidase-like 2; siRNA, small interfering RNA.

to further verify the expression status of the ccRCC cells, we chose four tumor cell lines (786-0, A-498, ACHN and Caki-1 cells) with different genetic backgrounds and derivations. Similarily, LOXL2 mRNA levels were significantly higher in the 786-0, A-498, ACHN and Caki-1 cells than in the HEK 293 cells (all $P < 0.05$; **Figure 1B**).

To investigate the functional role of LOXL2 in the ccRCC cells, we performed a loss-of-function study using the 786-0 cell line that was the most common cell line used in similar studies [22-24]. We evaluated the knockdown efficiency of LOXL2-siRNA in 786-0 cells using qRT-PCR and Western blot. mRNA and protein levels in the silenced cells were significantly lower than those in the control group ($P < 0.05$; **Figure 2**), indicating that lentiviral vector transfection with LOXL2-siRNA effectively reduced LOXL2 expression in the 786-0 cell line.

The proliferation of the 786-0 cells was decreased when LOXL2 expression was reduced

When the expression of LOXL2 was inhibited, the number of cells and the fold change in proliferation were markedly decreased in the 786-0 cells (**Figure 3**). These results suggested that the down-regulation of LOXL2 expression was related to cell proliferation.

LOXL2 expression down-regulation suppressed the ability of 786-0 cell growth when analyzed via BrdU labeling assay

A BrdU labeling assay was performed on the basis of thymine replacement competition in the S phase of the cell cycle. The down-regulation of LOXL2 expression significantly decreased the growth of 786-0 cells on day 4 ($P = 5.15 \times 10^{-5}$; **Figure 4**). It was proved that LOXL2 had an important role in promoting the growth of 786-0 cells.

LOXL2 expression down-regulation decreased the ability of 786-0 cell colony formation

LOXL2 expression down-regulation decreased the anchorage-independent growth ability of 786-0 in soft agar (**Figure 5A**). The cell colonies were reduced visually when observed using fluorescence and a lighted microscope (**Figure 5B** and **5C**). It was shown that the cell colony number was significantly reduced in 786-0 cells infected with LOXL2-siRNA ($P = 0.0028$; **Figure 5D**). These results suggest that LOXL2 is associated with the ability of 786-0 cell colony formation.

LOXL2 expression down-regulation led to cell cycle arrest in the 786-0 cell line

LOXL2 expression down-regulation significantly reduced the fraction of G1 phase cells ($P = 1.63 \times 10^{-5}$) and S phase cells ($P = 3.94 \times 10^{-4}$) in treated 786-0 cells compared to the controls. Meanwhile, the down-regulation of LOXL2 expression significantly increased the percentage of G2/M phase cells in treated 786-0 cells ($P = 2.71 \times 10^{-5}$; **Figure 6**). These results suggest that LOXL2 contributes to the cell phase arrest in ccRCC cells.

LOXL2 expression down-regulation promoted cellular apoptosis in the 786-0 cell line

The percentage of apoptotic cells was significantly increased in the 786-0 cells with reduced LOXL2 expression ($P = 5.84 \times 10^{-8}$; **Figure 7**) in

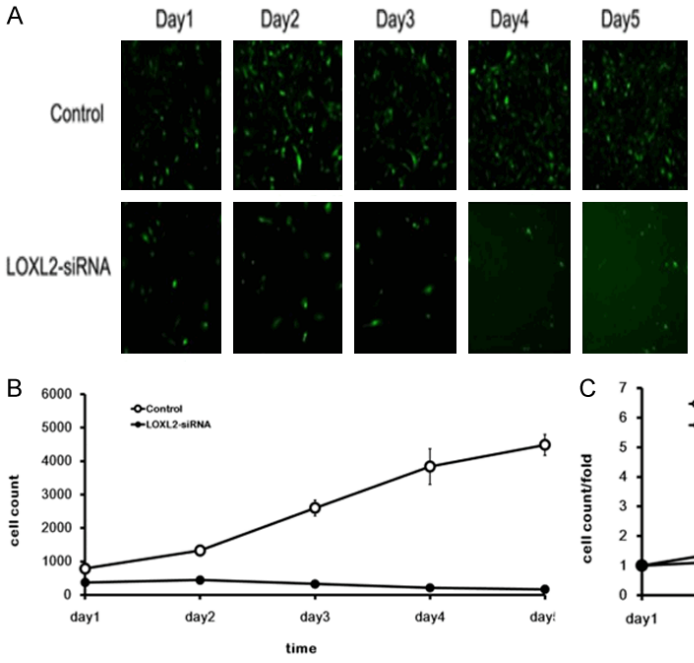

Figure 3. LOXL2 down-regulation reduced the proliferation of ccRCC cells when cells were observed by fluorescence microscopy (A). The curve of the number of cells (B) and fold change in proliferation (C) in 786-O cells treated with LOXL2-siRNA and the negative control group. LOXL2, lysyl oxidase-like 2; siRNA, small interfering RNA; ccRCC, clear cell renal cell carcinoma.

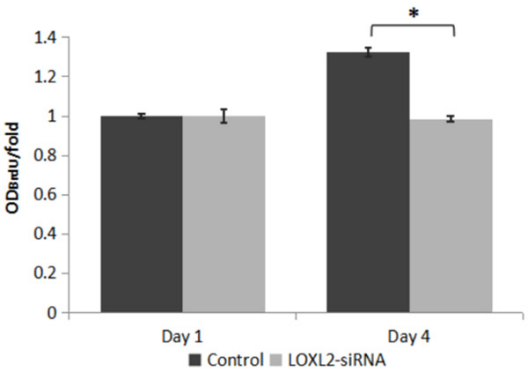

Figure 4. The effects of LOXL2 down-regulation on ccRCC cell absorbance. BrdU labeling was performed on the indicated days to demonstrate the absorbance of 786-O cells indicating that the growth ability of 786-O cells was suppressed when LOXL2 was down-regulated. *P < 0.01 vs. the control group. ccRCC, clear cell renal cell carcinoma; LOXL2, lysyl oxidase-like 2; OD, optical density; siRNA, small interfering RNA.

comparison with the controls. The results indicated that LOXL2 expression down-regulation accelerated apoptosis in the 786-O cells in vitro.

Discussion

This study first reported that LOXL2 was up-regulated in ccRCC tissues compared to adjacent normal renal tissues in the Chinese population. The effect of LOXL2 on ccRCC cell proliferation remains controversial in the literature. In our study, we proved in two different ways that LOXL2 down-regulation could effectively inhibit ccRCC cell proliferation. The effects of LOXL2 on the ccRCC cell cycle, cellular colony formation and apoptosis were rarely detected in the relevant studies [22-24]. Our results in vitro showed that tumor cell growth and colony formation ability were significantly decreased, and cell cycle arrest and cellular apoptosis were induced when LOXL2 expression was down-regulated in 786-O cells. It is considered that elevated LOXL2 levels may promote ccRCC tumorigenesis by enhancing tumor cell proliferation and the colony formation ability, getting more tumor cells into the G1 and S phases, and inhibiting cell apoptosis. LOXL2 may have the potential to be an alternative target for treating ccRCC.

To our knowledge, only a handful of published studies reported on LOXL2 expression status in ccRCC [22-24]. These studies were performed in the Japanese population, showing that LOXL2 was significantly elevated in ccRCC tissues [22-24]. Hase and colleagues [23] found ccRCC tissues had 4 times more LOXL2 mRNA compared to matched adjacent normal renal tissues. Similarly, we found that the expression of LOXL2 increased approximately 7.5-fold in tumor tissue from Chinese ccRCC patients in our study. The up-regulated expression of LOXL 2 could be associated with reduced tumor-sup-

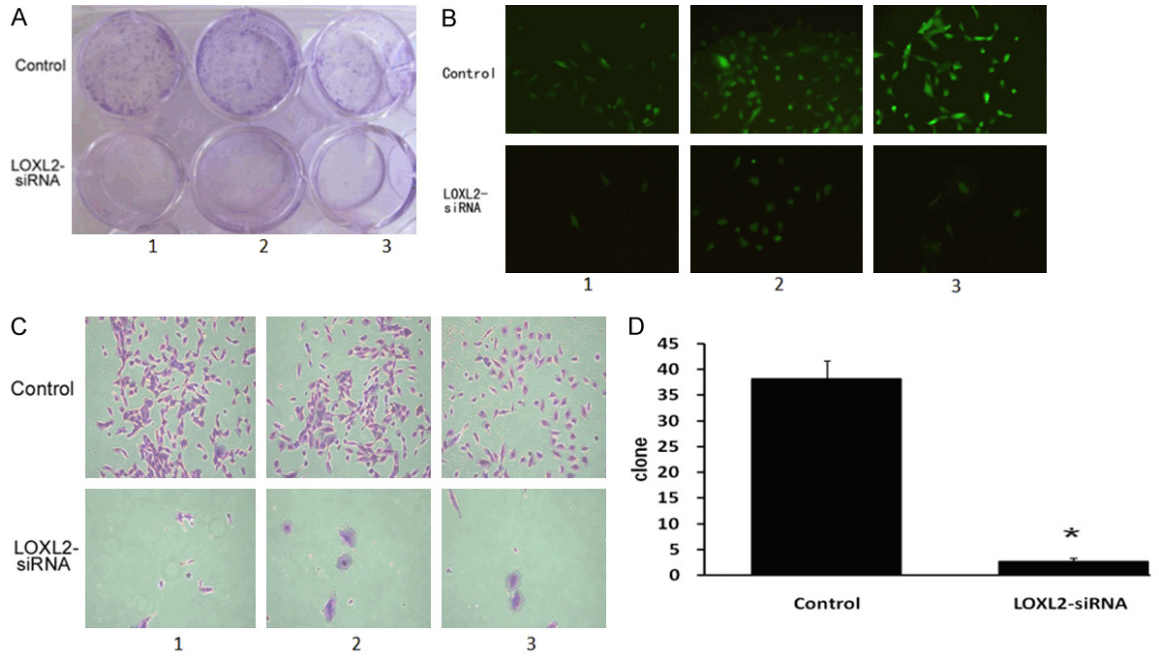

Figure 5. Effects of LOXL2 down-regulation on ccRCC cell colony formation. A. Representative images of the colony formation assay. B. Representative images of the cell colony observed using fluorescence microscopy in the six holes of the experimental and control groups. C. Representative images of cell colony stained with Giemsa in the six holes of the experimental and control groups. D. Histogram of colony formation quantification in 786-0 cells. *P < 0.01 vs. the control group. LOXL2, lysyl oxidase-like 2; siRNA, small interfering RNA.

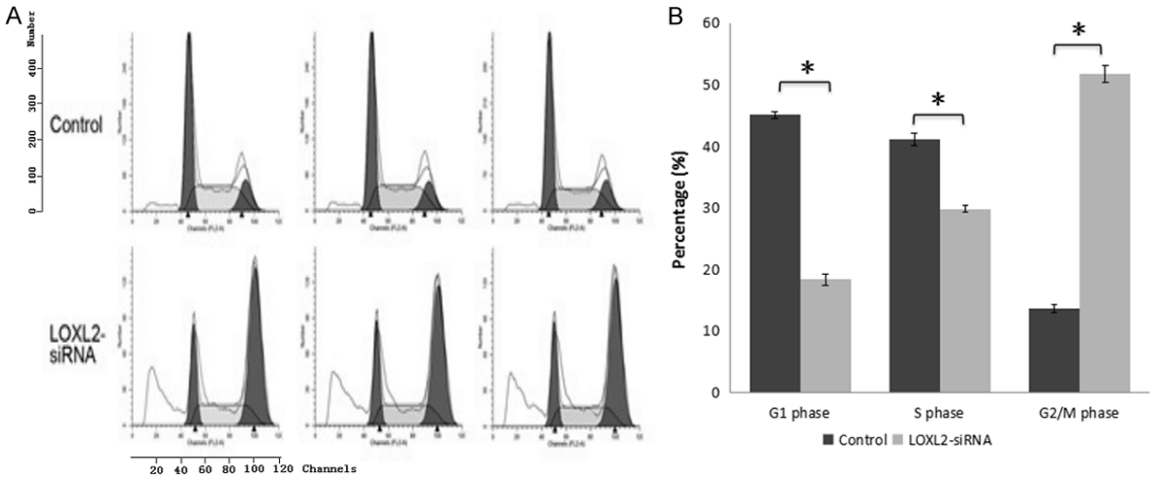

Figure 6. The effects of LOXL2 down-regulation on ccRCC cell cycle distribution. The 786-0 cells were significantly increased in G2/M phase and decreased in the G1 and S phases when LOXL2 expression was down-regulated. (A) Representative diagrams of flow cytometry and (B) histogram of the percentage of different cell cycle phases in the 786-0 cells. *P < 0.01 vs. the control group. ccRCC, clear cell renal cell carcinoma; LOXL2, lysyl oxidase-like 2; siRNA, small interfering RNA.

pressive microRNA-29s [22] and microRNA-26a/b [24] in ccRCC.

The effect of LOXL2 on ccRCC cell proliferation remains controversial [22-24]. Hase's research group found that down-regulating LOXL2 expression via LOXL2-siRNA transfection could inhibit tumor cell proliferation [23], but Nishikawa's group reported that cell proliferation could not be inhibited in all LOXL2-siRNA transfectant cells in comparison with the mock or miR-control transfectant cells [22]. The difference

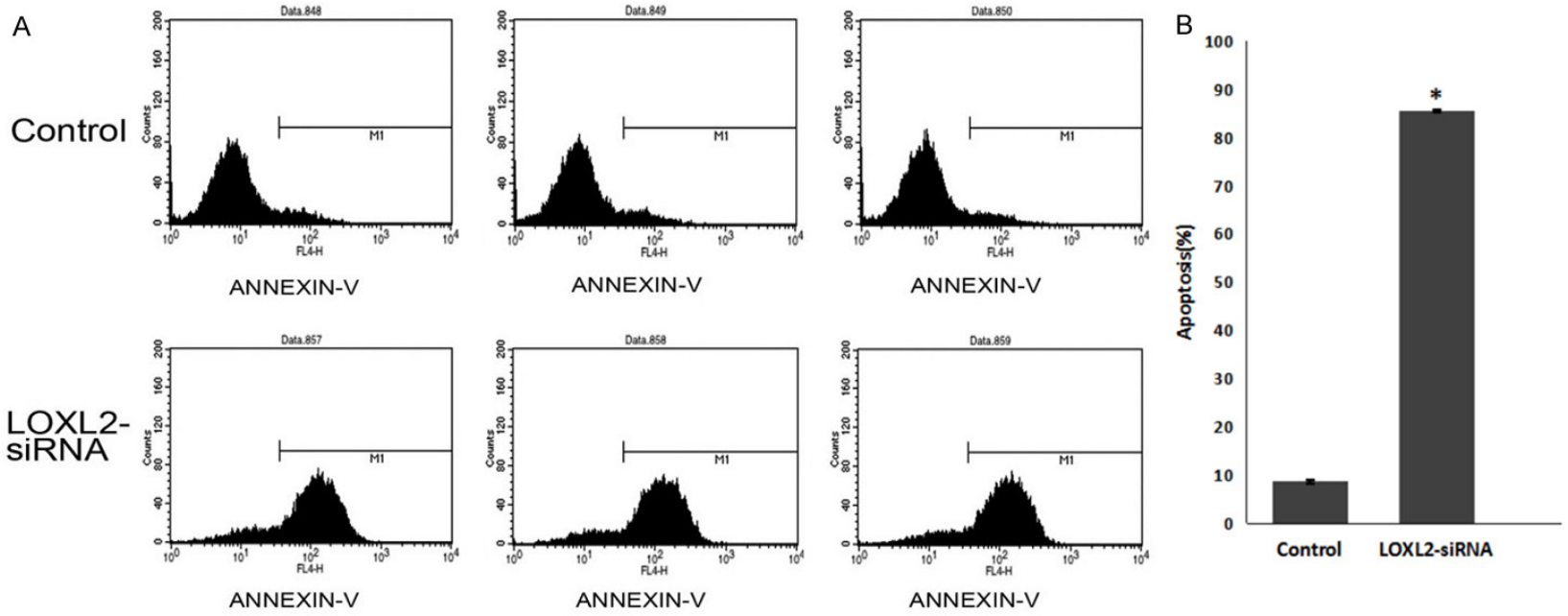

Figure 7. Effect of LOXL2 down-regulation on ccRCC cell apoptosis. The apoptosis of the 786-0 cells was significantly increased when LOXL2 was down-regulated. (A) Representative histograms of flow cytometry and (B) apoptotic cell rates in the 786-0 cells. *P < 0.01 vs. the control group. ccRCC, clear cell renal cell carcinoma; LOXL2, lysyl oxidase-like 2; siRNA, small interfering RNA.

between the two studies was the proliferation detection method. Based on this finding, we used the same method to down-regulate LOXL2 expression, and then we used two different detection methods to examine the status of tumor cell proliferation. As a result, the two experiments consistently showed that LOXL2 down-regulation could effectively inhibit ccRCC cell proliferation. In another study, Weidenfeld and colleagues demonstrated that LOXL2 provides cells with a proliferation ability in dormant tumor cells [28]. The precise regulatory mechanism that LOXL2 uses to affect cell proliferation in ccRCC remains unclear and requires further exploration [22].

In our study, cell cycle arrest was observed when LOXL2 expression was reduced in ccRCC cells, indicating a possible relationship between LOXL2 and the cell cycle, which was not explored in the previous studies [22-24]. In addition, cellular colony formation and apoptosis were also first reported to be related to LOXL2. The precise mechanisms regarding how LOXL2 regulates the tumor cell cycle, the colony formation ability, and apoptosis need to be further studied.

A high expression of LOXL2 may be associated with poor prognosis and has the potential to be a prognostic predictor of ccRCC, but no relevant study has been reported. Interestingly, the patient with lung metastasis nine months after the operation had the highest level of LOXL2 mRNA in our cohort. It was suggested that LOXL2 might be a potential prognostic factor for ccRCC. Hase reported that the LOXL2 level correlated with the pathological stage of ccRCC [23]. Renal cancer cell migration and invasion activity were enhanced by over-expressed LOXL2 that was linked to reduced microRNA-29s [22], microRNA-26a/b [24], and elevated integrin α5 and integrin β1 protein levels [23]. A high expression of LOXL2 has been reported to be associated with poor prognosis in other solid tumors. In pancreatic cancer, the relationship between LOXL2 over-expression and a low survival rate was demonstrated [29]. A three-gene signature comprising LOXL2, CDH1, and FN1 combined with TNM stage significantly enhanced the accuracy of evaluating esophageal squamous cell carcinoma prognosis [30]. A large sample cohort study will be necessary to investigate the relationship between the level of LOXL2 expression and ccRCC prognosis.

Conclusion

LOXL2 is highly expressed in Chinese patients with ccRCC and probably plays an important functional role in ccRCC tumorigenesis. However, the precise regulatory mechanisms need further exploration, and a larger sample cohort study will be needed to investigate the relationship between the LOXL2 expression level and ccRCC prognosis.

Acknowledgements

This work was supported by the Science and Technology Foundation of the Sichuan Province (2017SZ0123 to Zhi-hong Liu) and the 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University.

Disclosure of conflict of interest

None.

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