


ORIGINAL ARTICLE

Mitochondrial E3 ubiquitin ligase 1 promotes autophagy flux to suppress the development of clear cell renal cell carcinomas

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Abstract

Clear cell renal cell carcinoma (ccRCC) is one of the most common malignant tumors in the urinary system. Surgical intervention is the preferred treatment for ccRCC, but targeted biological therapy is required for postoperative recurrent or metastatic ccRCC. Autophagy is an intracellular degradation system for misfolded/aggregated proteins and dysfunctional organelles. Defective autophagy is associated with many diseases. Mul1 is a mitochondrion-associated E3 ubiquitin ligase and involved in the regulation of divergent pathophysiological processes such as mitochondrial dynamics, and thus affects the development of various diseases including *cancers*. Whether Mul1 regulates ccRCC development and what is the mechanism remain unclear. Histochemical staining and immunoblotting were used to analyze the levels of Mul1 protein in human renal tissues. Statistical analysis of information associated with tissue microarray and The Cancer Genome Atlas (TCGA) database was conducted to show the relationship between Mul1 expression and clinical features and survival of ccRCC patients. Impact of Mul1 on rates of cell growth and migration and autophagy flux were tested in cultured cancer cells. Herein we show that Mul1 promoted autophagy flux to facilitate the degradation of P62-associated protein aggresomes and adipose differentiation-related protein (ADFP)-associated lipid droplets and suppressed the growth and migration of ccRCC cells. Levels of Mul1 protein and mRNA were significantly reduced so that autophagy flux was likely blocked in ccRCC tissues, which is potentially correlated with enhancement of malignancy of ccRCC and impairment of patient survival. Therefore, Mul1 may promote autophagy to suppress the development of ccRCC.

KEYWORDS

autophagy, clear cell renal cell carcinoma, Mul1 protein, P62 protein, perilipin-2

Yaoji Yuan, Xiezhao Li, Yuyu Xu contributed equally to this work.

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1 | INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is one of the most common urinary tract malignant tumors with high morbidity and mortality. In the past two decades, the incidence of ccRCC worldwide has doubled, and the death toll is rising at a rate of 1% per year.¹ Development of ccRCC is difficult to predict, although epidemiological studies have found that tobacco consumption, obesity, hypertension and chronic nephropathy can induce ccRCC.¹⁻⁵ Localized ccRCC can be treated with partial, radical nephrectomy or thermal ablation.⁶⁻⁸ However, approximately 30% of patients with localized ccRCC who underwent surgical intervention developed further metastatic ccRCC (mccRCC) and required systemic treatment.⁷ Although many targeted drugs and biologicals for ccRCC are currently in the research and development stage, their efficacy remains to be explored. Hence, the identification of new and effective biomarkers is essential for a better understanding of the biological progression of ccRCC and to improve the diagnosis and prognosis of this cancer in clinical practice.

Autophagy is an intracellular degradation system that captures damaged or non-functional proteins and organelles and transports them to lysosomes for degradation.⁹ It plays an important homeostatic role in controlling the quality and quantity of proteins and organelles. Autophagy processes include cargo sequestration, transport to lysosomes, cargo degradation and recycling of degraded products, and the function of each different stage may be regulated differentially.¹⁰ LC3 functions as a core protein in autophagy and is mainly used for the recognition and recruitment of autophagy cargo. It elongates and seals the cargo and transports it to the autophagosomes.¹¹ At present, there are conflicting reports about the roles of autophagy in the development of cancer. Some studies related to breast cancer, colon cancer, lung cancer, prostate cancer and pancreatic cancer have shown that autophagy mainly promotes cancer development,¹²⁻¹⁶ whereas others related to liver cancer and ccRCC have shown the opposite results in that autophagy suppresses cancer development.¹⁷⁻¹⁹ Our previous study found that microtubule-associated protein family 1 (MAP1S) can promote autophagy clearance of lipid droplets and reduce DNA double-strand breaks and genome instability, consequently suppressing the development of ccRCC and promoting patient survival.¹⁹ Similarly, other groups have shown that autophagy can suppress ccRCC development.²⁰⁻²² Therefore, whether autophagy promotes or inhibits cancer development or whether autophagy has different regulatory mechanisms for cancer development in different tissues requires further investigation.^{9,23}

Mitochondrial E3 ubiquitin ligase 1 (Mul1), a multifunctional mitochondrial membrane-associated protein located on the outer membrane of the mitochondrion with two transmembrane domains and one Ring-finger domain,²⁴ regulates different biological processes such as mitochondrial dynamics, cell growth, apoptosis and mitophagy (autophagy degradation of mitochondria), through ubiquitination and SUMOylation.²⁵⁻²⁷ Mitophagy and mitochondrial dynamics cause frequent changes in both quantity and morphology of cells and organelles.²⁸ The imbalance of homeostasis is clearly associated with diseases such as Parkinson's disease, viral infection and

carcinomas.²⁹⁻³³ It was reported that Mul1 mediates the ubiquitination of Akt and HSPA5 to inhibit the development of head and neck cancer.^{32,33} Herein, we show that Mul1 promotes autophagy flux and suppresses ccRCC development with cell culture models and its loss of expression in ccRCC tissues from patients may lead to the blockade of autophagy flux, development of ccRCC and impairment of survival.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

Preliminary screening of levels of Mul1 protein by immunohistochemical analysis and immunoblot analysis with an antibody against Mul1 (ab84067) from Abcam were conducted on renal tissues from 11 patients and three patients diagnosed with ccRCC in the Fifth Affiliated Hospital, Guangzhou Medical University, respectively. Confirmation of levels of Mul1 protein by immunohistochemical analysis with the same antibody was conducted on a tissue microarray (TMA) from Xi'an Alenabio Co., Ltd (Cat No: PR803c) including 100 ccRCC tissues and 50 normal renal tissues with clinical information as summarized in Table 1. Further analyzing the relationship of levels of Mul1 mRNA with clinical features and survival times was conducted on information in a TCGA dataset including tumor tissues from 534 ccRCC patients with clinical features as summarized in Table 2. All procedures carried out in studies involving human patients were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The present study is a retrospective study in accordance with ethics review regulations and was reviewed and approved by the Ethics Committee, The Fifth Affiliated Hospital, Guangzhou Medical University (Guangzhou, China).

2.2 | Generation of stable cell lines

Cell lines HK2 (CC4008), 769-P (CC1504) and ACHN (CC1505) were purchased from Cellcook, Guangzhou. 769-P cells were transfected with control siRNA and Mul1-specific siRNAs Mul1-342, Mul1-486 and Mul1-1080 from Shanghai Genepharma or control lentiviral expression vector, and the vector with Mul1 was inserted between cut sites of restriction enzyme *EcoRI* and *BamHI* from Guangzhou HYY to select stable cell lines with the expression of Mul1 suppressed or elevated as confirmed by both quantitative RT-PCR and immunoblot analyses.

2.3 | Assays of cell growth rates and migration

Cell growth rates were carried out through the CCK-8 proliferation assay. The same number of cells were seeded and cultured in normal medium. After being cultured for 4 hours, 24 hours, 48 hours and 72 hours, cells were treated with 20 μ L CCK-8 solution for 2 hours and subjected to absorbance measurement at 450 nm by microplate reader. Cell migration rates were analyzed by wound-healing assay.

TABLE 1 Correlation of Mul1 expression with clinicopathological characteristics in patients with ccRCC (TMA)

Clinical features	TMA		
	Case	Mean ± SD	P-value
Tissue			
Cancer	100	0.91 ± 0.75	.000**
Normal	50	2.78 ± 0.91	
Age (y)			
<60	84	1.45 ± 1.14	.352
≥60	66	1.64 ± 1.26	
Gender			
Male	102	1.56 ± 1.17	.705
Female	48	1.48 ± 1.25	
Pathological grade			
G1	66	0.88 ± 0.75	.941
G2-G3	30	0.87 ± 0.73	
Clinical stage			
I	62	1.16 ± 0.73	.000**
II	38	0.50 ± 0.60	
Tumor invasion			
T1	62	1.16 ± 0.73	.000**
T2	38	0.50 ± 0.60	
Lymph node metastasis			
N0	100	0.91 ± 0.75	—
N1	0	—	
Distant metastasis			
M0	100	0.91 ± 0.75	—
M1	0	—	

Abbreviation: ccRCC, clear cell renal cell carcinoma; Mul1, mitochondrial E3 ubiquitin ligase 1; TMA, tissue microarray.

* $P < .05$; ** $P < .01$.

"—" mean: data cannot be analyzed.

Cells grown to full confluence on culture plates were treated with 4 $\mu\text{g}/\text{mL}$ mitomycin (Cat#: 10107409001) from Roche for 2 hours and scratched. Cells were continuously cultured and observed under a microscope to measure distances of cell migration at specific time points.

2.4 | Assays of the impact of Mul1 on autophagy

Stable cell lines with suppression or elevation of Mul1 were cultured to full confluence and treated with 10 $\mu\text{mol}/\text{L}$ Bafilomycin A1 (S1413) from Selleck for 6 hours. Cells were collected and lysed to prepare cell lysates. Cell lysates with the same amount of total protein as measured by the bicinchoninic acid (BCA) method were separated on SDS-PAGE and separated proteins were transferred to membranes to conduct immunoblot with Mul1 antibody as described above. LC3 antibody (ab192890) was from Abcam, and P62 antibody (#88588) was from CST. Levels of adipose differentiation-related protein (ADFP) in renal tissues from ccRCC patients from our hospital were

TABLE 2 Correlation of Mul1 expression with clinicopathological characteristics in patients with ccRCC (TCGA)

Clinical features	TCGA			
	Case	Low, n (%)	High, n (%)	P-value
Tissue				
Cancer	534	267 (50)	267 (50)	—
Normal	—	—	—	
Age (y)				
<60	246	144	102	.114
≥60	287	152	135	
Gender				
Male	345	215	130	.000**
Female	188	81	107	
Pathological grade				
G1-G2	242	115	127	.000**
G3-G4	283	179	104	
Clinical stage				
I-II	324	165	159	.005*
III-IV	206	129	77	
Tumor invasion				
T1-T2	342	178	164	.019*
T3-T4	191	118	73	
Lymph node metastasis				
N0	239	134	105	.409
N1	16	10	6	
Distant metastasis				
M0	421	230	191	.065
M1	79	51	28	

Abbreviation: ccRCC, clear cell renal cell carcinoma; Mul1, mitochondrial E3 ubiquitin ligase 1; TCGA, The Cancer Genome Atlas.

* $P < .05$; ** $P < .01$.

"—" mean: data cannot be analyzed.

analyzed by immunohistochemical staining with ADFP-specific antibody (cat# ab181452) from Abcam.

2.5 | Assignment of immunoreactivity scores and statistical analyses

To quantify the levels of Mul1 protein in renal tissues from ccRCC patients as shown by immunohistochemical staining, five random fields captured under microscope with a magnitude of 400-fold were selected. Percentage of positively stained cells to total cells was calculated and scored with the standard: <5% (0 points), 6%-25% (1 point), 26%-50% (2 points), 51%-75% (3 points), and >75% (4 points). Staining intensity was visually scored and stratified according to the following criteria: no staining (0 points), mild (1 point), moderate (2 points), and strong (3 points). The final immunoreactivity score of each case was calculated by adding the two scores based on the immunostaining percentage and the immunostaining intensity.

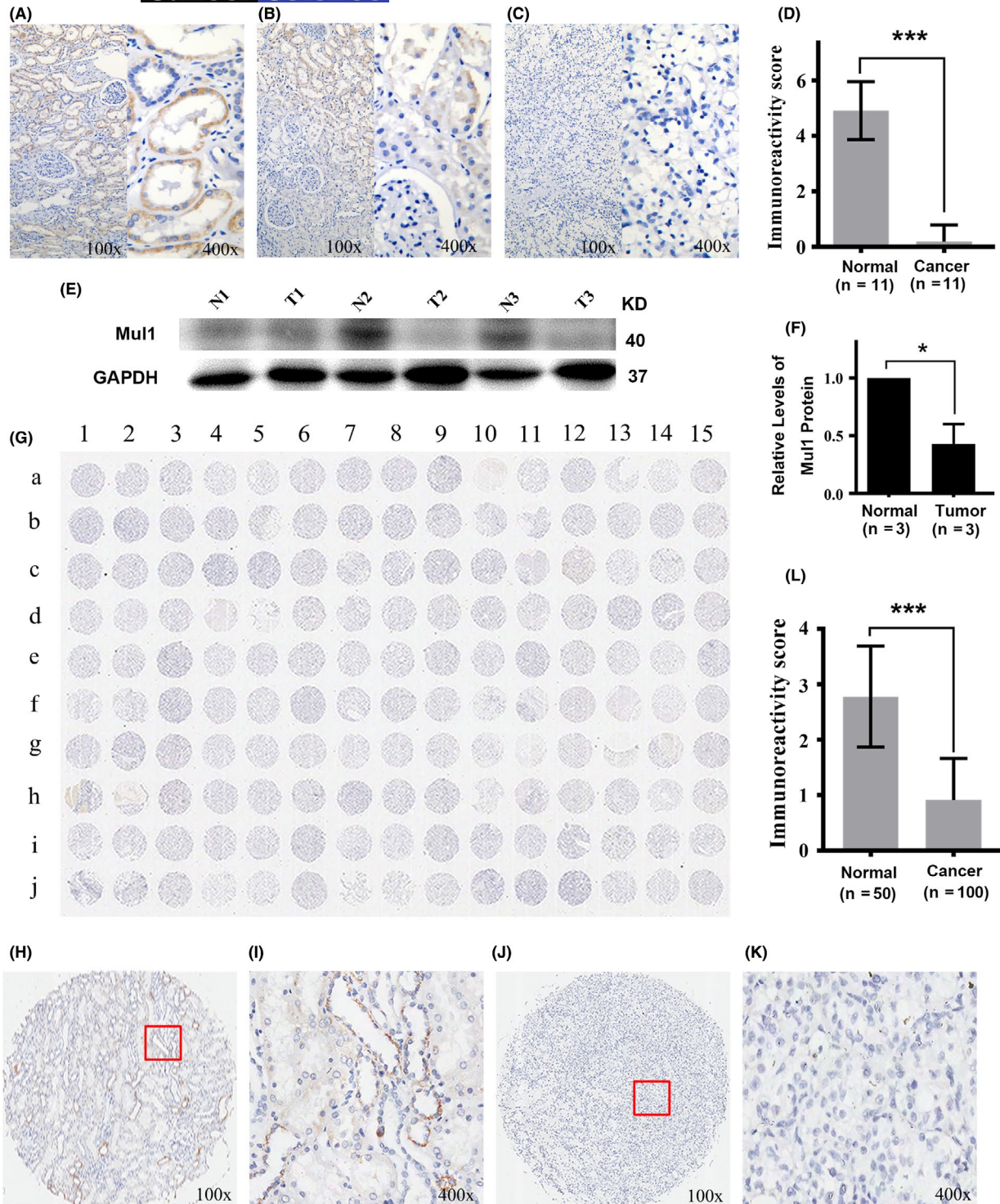
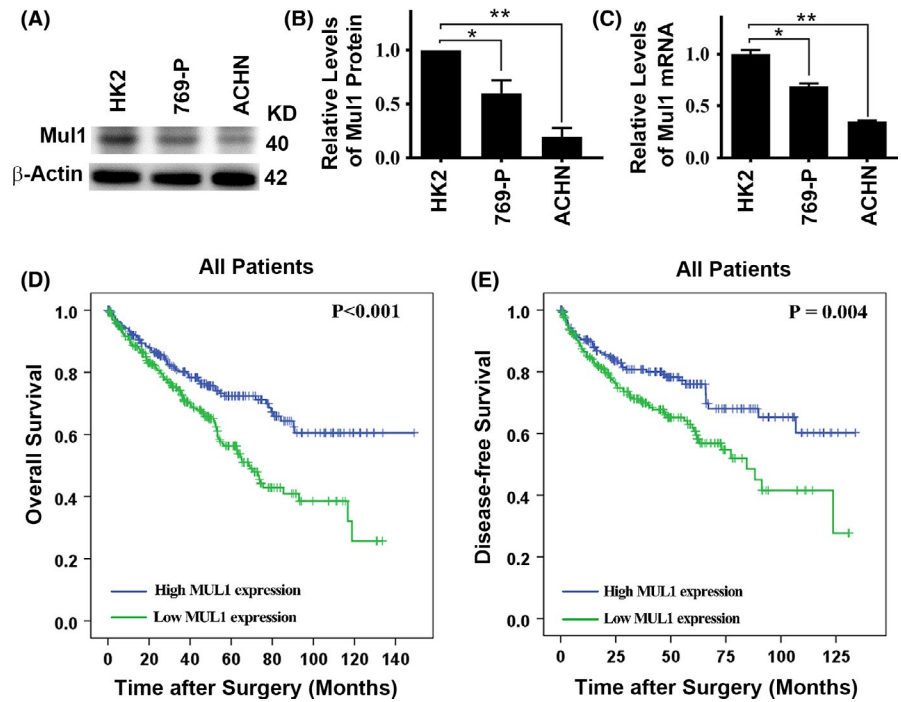


FIGURE 1 Expression of mitochondrial E3 ubiquitin ligase 1 (Mul1) protein in clear cell renal cell carcinoma (ccRCC) tissues and their adjacent normal tissues. (A–C) Representative images showing adjacent normal tissues expressing high levels (A), tissues near tumor tissues expressing intermediate levels (B) and ccRCC tissues expressing low levels of Mul1 (C) collected from 11 patients with ccRCC in our hospital. Left half is an image with low-resolution (100 \times) and right half with high-resolution (400 \times). (D) Plot showing a comparison of immunoreactivity scores of Mul1 between normal and cancer tissues as stated above. Mean and SD for normal tissues are 4.91 ± 1.04 and those for cancer tissues are 0.18 ± 0.60 . *** $P \leq .001$. Sample size is 11. (E, F) Immunoblot assays (E) and quantification (F) showing levels of Mul1 in tumor (T1, T2 and T3) and their respective adjacent normal tissues (N1, N2 and N3) from three ccRCC patients from our hospital. * $P \leq .05$. (G) Full immunohistochemical image showing expression levels of Mul1 in a tissue microarray containing 100 ccRCC and 50 non-cancerous tissues. (H–K) Representative images showing adjacent normal tissues (H, I) and ccRCC tissues (J, K) with low-resolution (100 \times) (H, J) or high resolution (400 \times) (I, K). (L) Plot showing a comparison of immunoreactivity scores of Mul1 between normal (n = 50) and cancer tissues (n = 100) as shown in G. Mean and SD for normal tissues are 2.78 ± 0.91 and those for cancer tissues are 0.91 ± 0.75 . *** $P \leq .001$

FIGURE 2 Relationship between levels of mitochondrial E3 ubiquitin ligase 1 (Mul1) mRNA in clear cell renal cell carcinoma (ccRCC) tissues and survival of ccRCC patients. (A,B) Representative immunoblot results (A) and quantification (B) showing levels of Mul1 protein in different types of renal cells. (C) Plot of relative levels of Mul1 mRNA in the same types of renal cells as shown in A,B. (D,E) Kaplan-Meier survival curves showing overall survival time (D) and disease-free survival (E) after surgery of ccRCC patients with high or low levels of Mul1 mRNA. Significance of difference between the two groups was estimated by chi-squared test. * $P < .05$; ** $P < .01$.



SPSS 22.0 software was used for statistical analysis. Survival was analyzed using the Kaplan-Meier method. Univariate analysis comparisons and multivariate survival comparisons were carried out using Cox proportional hazard regression models. Pearson's chi-squared tests (TCGA database) and Student's *t* tests (TMA database) were used to analyze the association of Mul1 expression with ccRCC clinicopathological characteristics. Differences were assigned as statistically significant when $P < .05$.

3 | RESULTS

3.1 | Expression of Mul1 protein is reduced in human ccRCC tissues

To probe the role of Mul1 protein in the development of ccRCC, we conducted preliminary screening of the levels of Mul1 protein in ccRCC tissues and their respective adjacent normal renal tissues from 11 patients collected in our hospital by immunohistochemical staining. It was found that the levels of Mul1 protein were high in the distal convoluted tubules and the proximal convoluted tubules of normal renal tissues but near negative in ccRCC tissue from the same patients (Figure 1A-D). The lower levels of Mul1 expression in ccRCC tissues compared to their respective normal renal tissues were further confirmed with tissues from three patients by immunoblot analysis (Figure 1E,F). To further verify the reduced expression of Mul1 in ccRCC tissues, we examined the levels of Mul1 in a commercially available TMA which contained 100 ccRCC and 50 adjacent non-cancerous tissues by immunohistochemical staining. Levels of Mul1 protein reflected by immunoreactivity score (IRS) were significantly lower in ccRCC tissues than in normal tissues (Figure 1G-L, Table 1). Therefore, the expression of Mul1 protein is dramatically reduced in ccRCC tissues.

TABLE 3 Prognostic value of Mul1 expression on disease-free survival by Cox proportional hazards model

Variable	Disease-free survival	
	HR (95% CI)	P-value
Univariate analysis		
Age, y (≥ 60 vs < 60)	1.820 (1.330-2.491)	.000**
Gender (Male vs Female)	1.058 (0.778-1.440)	.719
Tumor invasion (T1-T2 vs T3-T4)	3.164 (2.339-4.281)	.000**
Lymph node stage (N0 vs N1)	3.386 (1.797-6.377)	.000*
Pathological grade (G1-G2 vs G3-G4)	2.612 (1.860-3.669)	.000*
Clinical stage (S1-S2 vs S3-S4)	3.853 (2.810-5.283)	.000**
Distant metastasis (M0 vs M1)	4.348 (3.187-5.930)	.000**
Mul1 expression (low vs high)	0.552 (0.402-0.757)	.000**
Multivariate analysis		
Mul1 expression (low vs high)	0.663 (0.479-0.918)	.013*
Tumor invasion (T1-T2 vs T3-T4)	2.308 (1.672-3.186)	.000**
Age, y (≥ 60 vs < 60)	1.605 (1.168-2.208)	.004**
Pathological grade (G1-G2 vs G3-G4)	1.765 (1.227-2.540)	.002**

Abbreviation: CI, confidence interval; HR, hazard ratio; Mul1, mitochondrial E3 ubiquitin ligase 1.

* $P < .05$; ** $P < .01$.

3.2 | Reduced expression of Mul1 predicts poor prognosis of human patients with ccRCC

We examined the levels of Mul1 mRNA and protein in cultured cells including HK-2 (human kidney 2), an immortalized proximal

tubular cell line derived from a normal kidney, cell line 769-P from primary clear cell adenocarcinoma, and ACHN from a patient with metastatic renal adenocarcinoma. The levels of Mul1 in cancer cells 769-P and ACHN were lower than those in normal renal cell HK-2 as indicated by levels of both protein and mRNA (Figure 2A-C). The consistent low expression of both Mul1 mRNA and protein in ccRCC tissues prompted us to analyze the data deposited in TCGA database which contains information on the levels of Mul1 mRNA in cancer tissues from 534 ccRCC patients (Table 2). In agreement with results related to protein levels, patients diagnosed with higher clinical stage and higher degree of tumor invasion expressed lower levels of Mul1 mRNA and protein (Tables 1 and 2). Further analyses indicated that patients with lower levels of Mul1 mRNA had significantly poorer overall and disease-free survival than those with higher levels of Mul1 mRNA (Figure 2D,E). In addition, univariate analysis and further multivariate analysis indicated that lower levels of Mul1 mRNA but higher degree of tumor invasion and pathological grade showed higher hazard ratio to disease-free survival (Table 3). Therefore, reduced levels of Mul1 predict more malignant ccRCC and poor prognosis of ccRCC patients.

3.3 | Mitochondrial E3 ubiquitin ligase 1 inhibits the growth and migration of ccRCC cells

In order to test the roles of Mul1 in tumorigenesis, we generated stable cell lines with reduced or overexpressed levels of Mul1 in the background of cancer cell 769-P. Treating 769-P cells with two types of Mul1-specific siRNA molecule led to successful suppression of Mul1 protein (Figure 3A,B), whereas adding a plasmid carrying the Mul1 gene did not increase the levels of Mul1 protein (Figure 3C,D). Further measurements with RT-PCR indicated that the treatment with siRNA led to a significant reduction whereas

treatment with plasmid led to a significant increase in the levels of Mul1 mRNA (Figure 3E,F).

To test the impact of Mul1 on growth and migration, we conducted CCK-8 proliferation assay and wound-healing experiments using stable cell lines. Suppressing the expression of Mul1 led to a significant increase in cell growth rates (Figure 4A). Because of the inefficiency to increase the levels of Mul1 protein by overexpression, cells transfected with Mul1 expression plasmid did not show any significant impact on growth as expected (Figure 4B). Similarly, suppressing expression of Mul1 promoted cell migration (Figure 4C,D), but the overexpression of Mul1 did not change the migration rates as expected (Figure 4C,E). In general, Mul1 inhibits the growth and migration of ccRCC cells.

3.4 | Mitochondrial E3 ubiquitin ligase 1 promotes autophagy flux in ccRCC

Although it was observed that cells transfected with Mul1-expressing plasmid showed higher levels of Mul1 mRNA (Figure 3F) but did not show increased levels of Mul1 protein (Figure 3C,D), levels of Mul1 protein did increase in cells expressing Mul1 in the presence of lysosomal inhibitor Bafilomycin A1 (Figure 5A,B). Levels of Mul1 protein in the presence of Bafilomycin A1 were reduced as expected when cells were treated with Mul1-specific siRNA (Figure 5C,D). These results suggested that overexpressed Mul1 protein was degraded immediately and efficiently through the lysosomal system.

To further determine the cellular function of Mul1, we examined the levels of autophagy marker LC3-II and P62 after bafilomycin treatment. Treating cells with Mul1-specific siRNA to reduce the levels of Mul1 led to a reduction in autophagy flux as reflected by the reduced levels of LC3-II in the presence of Bafilomycin A1 and an accumulation of aggresome marker P62 in the absence of

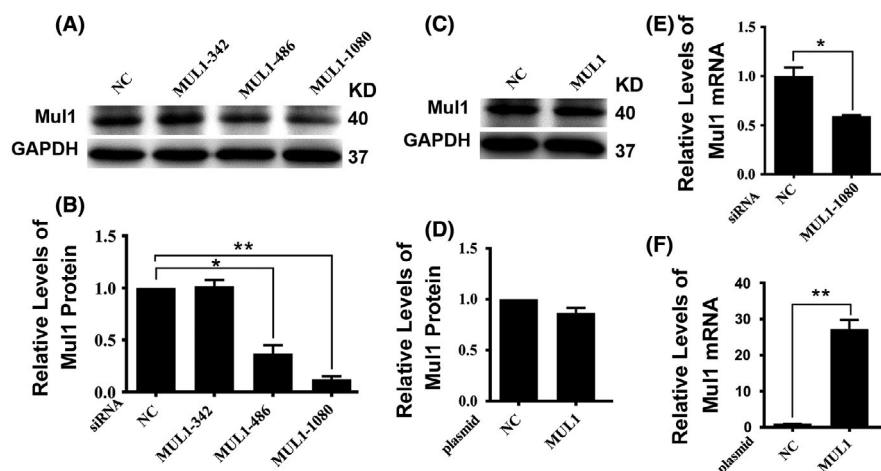
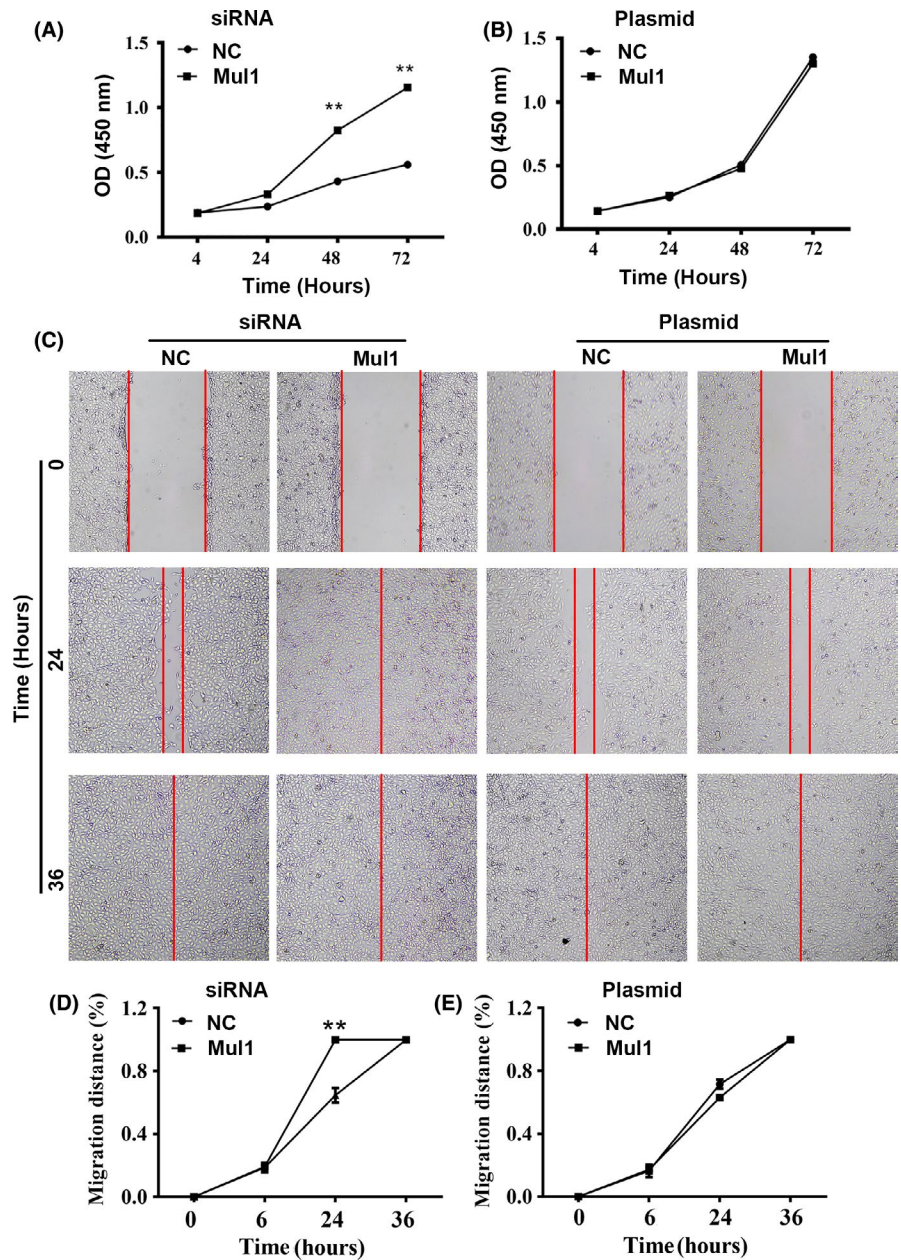


FIGURE 3 Expression of mitochondrial E3 ubiquitin ligase 1 (Mul1) in engineered 769-P cells. (A-D) Representative immunoblot results (A,C) and their respective quantification (B,D) showing levels of Mul1 protein in control cells (NC) and stable cell lines transfected with different Mul1-specific siRNAs (A,B) or control cells (NC) and stable cell lines transfected with Mul1 expressing plasmid (C,D). (E,F) Plots showing a comparison of levels of Mul1 mRNA between control (NC) and stable cell line with siRNA 1080 (E) and between control (NC) and stable cell line with Mul1 plasmid (F). * $P < .05$; and ** $P < .01$

FIGURE 4 Impact of mitochondrial E3 ubiquitin ligase 1 (Mul1) on growth and migration of clear cell renal cell carcinoma (ccRCC) cells. (A,B) Plots showing a comparison of growth rates between control (NC) and cells with Mul1-specific siRNA (A) or between control (NC) and cells with Mul1 plasmid (B). (C-E) Representative images (C) and plots (D,E) showing a comparison of migration distances between cells as stated in A (D) or B (E). $**P \leq .01$



Bafilomycin A1 (Figure 5E-G). Overexpressing Mul1 caused an increase in autophagy flux as reflected by the increased levels of LC3-II in the presence of Bafilomycin A1 and a dramatic reduction of aggresomes as reflected by the reduced levels of P62 in the presence of Bafilomycin A1 (Figure 5H-J). Autophagy defects in the degradation of ADFP-associated lipid droplets may enhance the initiation and development of ccRCC and reduce the survival of ccRCC patients.¹⁹ Mul1 deficiency in ccRCC tissues predicted a blockade of autophagy degradation of lipid droplets and an accumulation of their associated ADFP. Examination of levels of ADFP by histo-immunochemical staining showed that levels of ADFP were significantly higher in ccRCC tissues than in adjacent normal or fibrotic tissues (Figure 5K-N). Therefore, Mul1 promotes autophagy flux and its loss in tumor tissues may promote the development of ccRCC.

4 | DISCUSSION

As a common urological malignancy, based on epidemiological data, ccRCC was shown to be caused by obesity, smoking, hypertension, chronic kidney disease and type 2 diabetes, suggesting the potential to prevent the development of ccRCC.²⁻⁵ Established surgical treatments have shown good results for the treatment of localized ccRCC.⁶⁻⁸ However, the treatment for postoperative recurrence or concurrent metastasis of mcrRCC still lacks any specific therapeutic drug that can improve the survival of ccRCC patients.

Currently, the relationship between autophagy and cancer is still unclear. In general, basal autophagy flux inhibits early tumor development by maintaining homeostasis and suppressing genomic damage events.³³ Because the metabolic and biosynthesis requirements of tumor cells are significantly increased, it seems that cancer cells

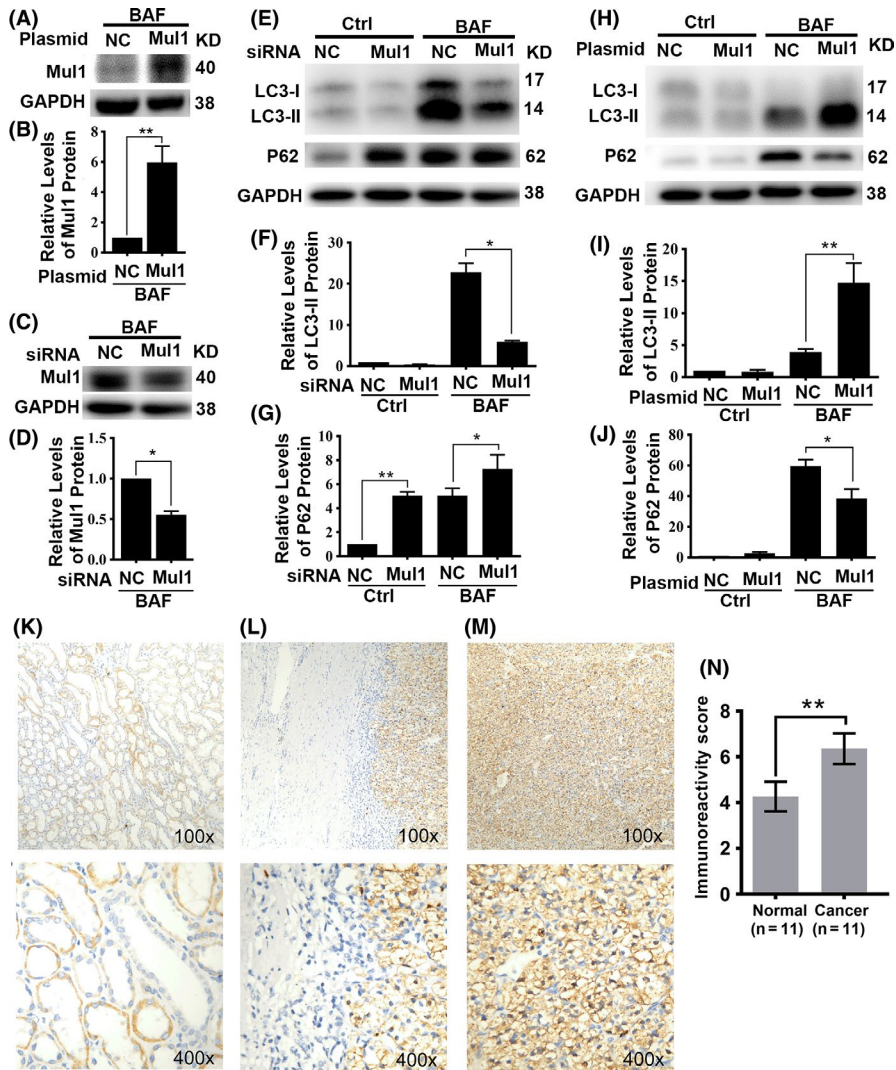


FIGURE 5 Impact of mitochondrial E3 ubiquitin ligase 1 (Mul1) on autophagy flux. (A–D) Representative immunoblots (A, C) and plots (B, D) showing levels of Mul1 protein in cells with Mul1 plasmid (A, B) or siRNA (C, D) in the presence of Bafilomycin A1 (BAF). (E–J) Representative immunoblots (E, H) and plots (F, G, I, J) showing levels of LC3-II (F, I) or P62 (G, J) protein in cells with siRNA (E–G) or Mul1 plasmid (H–J) in the absence (Ctrl) or presence of Bafilomycin A1 (BAF). * $P \leq .05$; and ** $P \leq .01$. (K–M) Representative images showing adjacent normal tissues expressing normal levels (K), fibrotic tissues near clear cell renal cell carcinoma (ccRCC) tissues expressing low levels (L) and ccRCC tissues expressing high levels of adipose differentiation-related protein (ADFP) (M) collected from 11 patients diagnosed with ccRCC in our hospital. (N) Plot showing a comparison of immunoreactivity scores of ADFP between normal and cancer tissues from ccRCC patients as stated above. ** $P \leq .01$. Sample size is 11

depend more on autophagy than do normal cells. Therefore, some suggest that autophagy promotes the development of advanced tumors.³⁴ However, the latest research findings show that the development of cancer is often accompanied by the decline or loss of autophagy. When autophagy flux is blocked, cancer cells accumulate more genomic errors and become more malignant.³⁵ Our previous results showed that promoting autophagy is beneficial to inhibit the development of hepatocellular carcinoma and ccRCC and to improve patient survival.³⁵ It was found that autophagy flux was relatively reduced in ccRCC and negatively correlated with tumor clinical stage and pathological grade.¹⁷

Mitochondrial E3 ubiquitin ligase 1 was originally discovered as an E3 ligase of ubiquitination. It promotes autophagy degradation of mitochondria by interacting with a specific E2 ubiquitination-binding enzyme and autophagy-related protein GABARAP,²⁵ or mediating the ubiquitination of autophagy upstream protein ULK1.²⁷ Although Mul1 protein was immediately degraded potentially because of the Mul1-enhanced autophagy flux, the autophagy flux is actually increased in cells overexpressing Mul1 as confirmed by increased levels of Mul1 mRNA. However, the reduced expression of Mul1 in ccRCC tissues compared to their adjacent normal renal tissues likely suggests true

reductions in both mRNA and protein of Mul1 and autophagy flux. Loss of Mul1 expression and blockade of autophagy flux lead to the accumulation of P62-associated protein aggregates. P62 acts as a selective autophagy receptor protein to recruit ubiquitinated autophagy substrates in autophagosomes and transfers them to lysosomes for degradation.^{36,37} Impairment of autophagy flux enhances ccRCC development by regulating degradation of hypoxia-inducible factor 2 α through P62.³⁸ Suppression of Mul1 led to the enhancement of proliferation and migration of ccRCC cells, promotion of cancer malignancy and reduction of ccRCC patient survival. However, increased expression of Mul1 did not impact cell growth and migration but did enhance autophagy flux, suggesting that not the growth and migration of tumor cells, but the malignancy of ccRCC and its associated patient survival are directly related to autophagy flux. In summary, Mul1 promotes autophagy flux and its loss is associated with blockade of autophagy flux, accumulation of protein aggregates, enhancement of ccRCC malignancy, and impairment on patient survival. The observations in the present study suggest that Mul1 might exert anti-tumorigenic roles in certain stages of ccRCC development, although in vivo studies are required to reach a final conclusion.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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