LOXL2 Status Correlates with Tumor Stage and Regulates Integrin Levels to Promote Tumor Progression in ccRCC

Hiroaki Hase¹, Kentaro Jingushi¹*, Yuko Ueda¹, Kaori Kitae¹, Hiroshi Egawa¹, Ikumi Ohshio¹, Ryoji Kawakami¹, Yuri Kashiwagi¹, Yohei Tsukada¹, Takumi Kobayashi¹, Wataru Nakata², Kazutoshi Fujita², Motohide Uemura², Norio Nonomura² and Kazutake Tsujikawa¹

¹Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan and

²Department of Urology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, 565-0871, Japan

Conflict of interest disclosure statement: The authors have no conflict of interest to declare.
Running title: LOXL2 regulates integrin α5/β1 in ccRCC cells

*Corresponding author: Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan. Tel: +81-6-6879-8192; Fax: +81-6-6879-8190; Email: jingushi-kk@phs.osaka-u.ac.jp

Key words: LOXL2, integrin α5, integrin β1, focal adhesion, clear cell renal cell carcinoma

Abbreviations: ccRCC: clear cell renal cell carcinoma, LOXL2: lysyl oxidase-like protein 2, ITGA5: integrin α5, ITGB1: integrin β1, FAK: focal adhesion kinase, ECM: cell-extracellular matrix, EMT: epithelial-mesenchymal transition
Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common histologically-defined subtype of renal cell carcinoma (RCC). To define the molecular mechanism in the progression of ccRCC, we focused on LOX-like protein 2 (LOXL2), which is critical for the first-step in collagen and elastin crosslinking. Using exon array analysis and quantitative validation, LOXL2 was shown to be significantly upregulated in clinical specimens of human ccRCC tumor tissues, compared to adjacent non-cancerous renal tissues, and this elevated expression correlated with the pathological stages of ccRCC. RNAi-mediated knockdown of LOXL2 resulted in marked suppression of stress-fiber and focal adhesion formation in ccRCC cells. Moreover, LOXL2 siRNA knockdown significantly inhibited cell growth, migration, and invasion. Mechanistically, LOXL2 regulated the degradation of both integrins \( \alpha_5 \) (ITGAV5) and \( \beta_1 \) (ITGB1) via protease- and proteasome-dependent systems. In clinical ccRCC specimens, the expression levels of LOXL2 and integrin \( \alpha_5 \) correlated with the pathological tumor grades. In conclusion, LOXL2 is a potent regulator of integrin \( \alpha_5 \) and integrin \( \beta_1 \) protein levels and functions in a tumor-promoting capacity in ccRCC.
Implications

This is the first report demonstrating that LOXL2 is highly expressed and involved in ccRCC progression by regulating the levels of integrins α5 and β1.
Introduction

Renal cell carcinoma (RCC) is the leading cause of death among urological malignancies, and clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of RCC. Early-stage ccRCC is usually curable by clinical surgery, but a large number of early stage ccRCC cases are asymptomatic, with approximately one-third of all patients presenting with locally metastatic cancer at the time of diagnosis (1). Therefore, a better understanding of the molecular mechanisms of ccRCC progression is crucial for the discovery of novel prognostic markers and targeted therapies.

A genetic hallmark of ccRCC is the inactivation of the von Hippel Lindau (VHL) tumor suppressor gene. VHL functions as a part of an E3 ubiquitin ligase complex that controls protein levels of hypoxia inducible factor (HIF), a transcription factor and master regulator of the cellular response to hypoxia. When VHL is absent, HIF accumulates, even under normoxic conditions, and inappropriately transactivates target gene expression. Because HIF target genes are potently tumorigenic, HIF target
gene expression is considered the primary orchestrator of VHL-deficient ccRCC tumor progression. Because of this, many approaches have been attempted that target downstream molecules of HIF for ccRCC therapeutics. Molecular targeted drugs for ccRCC therapy are developed the basis of such ideas, but these agents only delay disease progression and are not curative (2). Therefore, it is necessary to clarify critical molecular mechanisms in ccRCC tumorigenesis and progression.

LOXL2 is a member of the lysyl oxidase gene family, comprising five members, LOX and four LOX-like proteins (LOXL1–4), and functions both intracellularly and extracellularly. Secreted LOXL2 catalyzes the covalent crosslinking of the collagen and elastin component side chains via its lysyl oxidase activity, thereby stabilizing the proteins in the extracellular matrix (ECM) (3). On the other hand, intracellular LOXL2 downregulates E-cadherin expression by cooperating with the transcriptional factor Snail (4, 5) or regulating the methylation status of the E-cadherin gene (6), thereby promoting epithelial-mesenchymal transition (EMT). Moreover, LOXL2 was recently shown to deaminate lysine 4 in histone H3 to transcriptionally control the E-cadherin gene (CDH1) (7). Through these mechanisms, LOXL2 is
involved in cancer progression. However, to our knowledge, no reports have investigated the expression and function of LOXL2 in ccRCC.

Integrins are ubiquitously expressed transmembrane receptors of at least 24 heterodimers composed of 18 α-subunits and eight β-subunits (8). Integrins function in both cell-cell interactions, through binding to cadherins, and cell-extracellular matrix (ECM) interactions, involving fibronectin, collagen, and laminin (9, 10). Hence, deregulated integrin expression or function correlates with cancer progression by enhancing cancer cell invasion, metastasis, and survival. For example, loss of integrin β1 expression blocks tumor progression in different tumor models (11, 12), and increased expression of integrin α5/β1 is associated with poor prognosis in early-stage non-small cell lung cancer (13). However, the regulation mechanisms of integrin expression remain unclear in cancer cells.

In the present study, we found that LOXL2 was significantly upregulated in ccRCC tissues compared to normal renal tissues. LOXL2 knockdown suppressed proliferation, migration, and invasion by inhibiting focal adhesion complex and stress fiber formation in ccRCC cells. We also clarified that intracellular LOXL2 is involved
in the stabilization of integrin α5 and integrin β1 through suppression of the protease and proteasome systems. Moreover, we show that the protein levels of LOXL2 and integrin α5 were significantly increased in ccRCC specimens. To the best of our knowledge, this is the first report showing that the highly expressed LOXL2 is involved in ccRCC progression by regulating the expression of integrins α5 and β1.
Materials and methods

Chemicals and antibodies

The polyclonal anti-LOXL2 antibody was purchased from Abcam (Cambridge, MA, USA). The polyclonal anti-FAK, anti-integrin α5, and anti-integrin β1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-phospho-FAK (Tyr576) and anti-β-actin antibodies were purchased from Sigma (St Louis, MO, USA). Leupeptin was purchased from Tocris Bioscience (United Kingdom), and MG132 was purchased from Sigma.

Clinical specimens

ccRCC clinical specimens were obtained from patients during primary curative resection at Osaka University Medical Hospital in Japan. Tumor-associated normal renal tissues were also obtained from a subset of these patients where possible. Prior written and informed consent was obtained from each patient, and the study was approved by the ethics review board of Osaka University Medical Hospital. Following
excision, tissue samples were immediately immersed in RNAlater (Qiagen, Germantown, MD, USA) and stored at -20°C until RNA extraction. mRNA was purified using the RNeasy mini kit (Qiagen). An exon array was conducted using 4 paired samples. LOXL2 expression was validated by qPCR using 31 paired ccRCC samples and 90 tumor specimens. Clinical and pathological data for the clinical samples are presented in Tables 1 and 2 for exon array analysis and qPCR analysis, respectively.

**Exon array analysis**

Total RNA (100 ng) was subjected to cDNA synthesis using the Ambion WT expression kit (Life Technologies, Carlsbad, CA). The obtained 5.5 μg of cDNA were biotinylated and then fragmented with the GeneChip WT terminal labeling kit (Affymetrix, Santa Clara, CA). After creating the hybridization cocktail, biotinylated cDNAs were hybridized with the GeneChip human Exon 1.0 ST array (Affymetrix) for 17 h. After hybridization, the GeneChip was washed and stained using the GeneChip hybridization wash and stain kit (Affymetrix) and the GeneChip Fluidics Station (Affymetrix). The GeneChip was then scanned by the GeneChip Scanner 3000 (Affymetrix). Gene Spring
12.1 (Agilent Technologies, Mississauga, ON, USA) was used for the data analysis.

**Cell culture**

The four human ccRCC cell lines 786-O, Caki-1, Caki-2, and ACHN were cultured in RPMI1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 0.1 μg/mL streptomycin. The HEK 293T cell line was cultured in DMEM medium (Wako) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 0.1 μg/mL streptomycin. Normal human renal epithelial cells (Kurabo, Osaka, Japan) were cultured with RenaLife BM medium supplemented with RenaLife LifeFactor (Kurabo).

**qPCR for LOXL2 and integrin α5 mRNA expression**

Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). PrimeScript RT reagent Kit (Takara, Shiga, Japan) was used to prepare cDNA from 500 ng total RNA. The light cycler (Roche, Rotkreuz, Switzerland) was used for qPCR analysis. Thermal cycling conditions included an initial step at 95°C for 30 s and 50
cycles of 94°C for 15 s, 60°C for 10 s, and 72°C for 15 s. Primer sequences for gene amplification were as follows: LOXL2 forward, 5′-TTCCCTGGGGAGAGGGACATACA-3′, and reverse, 5′-TTCTTCATGGGGTCCAGTGACA-3′; integrin α5 forward, 5′-GGCAGCTATGGCGTCCCACTGTGG-3′, and reverse, 5′-GGCATCAGAGGTGGCTGGAGGCTT-3′; and integrin β1 forward, 5′-GACAAATTACCCCAAGCCGGTCCAAC-3′, and reverse, 5′-GGCCAATAAGAACAATTCAGCAACCA-3′.

siRNA and DNA transfection

siRNA duplexes used to downregulate LOXL2 and Integrin α5 mRNA expression (LOXL2 siRNA #1: GCCACATAGGTGGTTCCTTCATT, LOXL2 siRNA #2: AGTAAAGAAGCCTGCGTGGTCTT, Integrin α5 siRNA #1: CAGAUAACUUCACCCGAAUTT, and Integrin α5 siRNA #2: GCAGAUUGCAGAAUCUUAUTT) were purchased from Sigma Aldrich. The negative control siRNA duplex (AGTAAAGAAGCCTGCGTGGTCTT) was purchased from
B-Brige (Cupertino, CA). For all siRNA transfection studies, 786-O cells were seeded at a density of $2 \times 10^4$ cells/cm² and 10 nM siRNA was transfected using Lipofectamine RNAiMAX reagent (Life Technologies, Carlsbad, CA). For DNA transfection, $2 \times 10^4$ HEK 293T cells were seeded in a 48-well plate 24 h before transfection. DNA transfection was performed using HilyMax transfection reagent (Wako).

**Western blotting**

Samples were separated by 7.5% to 15% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride membrane using a semidry transfer system (Bio-Rad, Hercules, CA). Immunoreactive proteins were visualized with ECL Prime western blotting detection reagent (GE Healthcare, Buckinghamshire, United Kingdom). Densitometric analysis was performed using the NIH Image J software.

**Fluorescence microscopy**

Cells, plated on coverslips, were incubated for the indicated times and washed with
phosphate-buffered saline (PBS). The cells were fixed and permealized in 4% formaldehyde for 15 min at room temperature and then washed twice with PBS. After blocking with 5% skim milk for 1 h, the coverslips were incubated with primary antibody at 4°C. The coverslips were washed twice with PBS and incubated with fluorochrome-conjugated secondary antibody for 1 h at room temperature. The cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Construction of LOXL2 expression vector

The LOXL2 sequence was PCR-amplified from human genomic DNA followed by cloning into the pDEST26 vector (Invitrogen). The primer sequences for gene amplification were as follows: forward, 5′-CACCATGGAGAGGCCTCTGTGCTCCCACC-3′, and reverse, 5′-TCG AGG ATC CGG CCC AAT CCC TGC TTC CAC T-3′.

Wound healing assay
Cell migration was examined using a wound healing assay. 786-O cells transfected with siRNAs were seeded in a 24-well plate (1.0 × 10^5 cells/well) and incubated for 72 h. A wound was created in a monolayer of ~90% confluent 786-O cells, using a sterile 1 ml pipette tip. Cell migration pictures were recorded at 0 h and 12 h, using an Olympus IX71 fluorescence Microscope.

**Cell proliferation and cell invasion assay**

Cell proliferation and cell invasion assay were each carried out using E-plate and CIM-plate respectively and analysed by xCELLigence system (Roche) according to the manufacturer.

**Cell membrane biotinylation**

The cell membranes of 786-O cells that were transfected with LOXL2 siRNA or control siRNA for 24 h were biotinylated with membrane-impermeable EZ-LINK Sulfo-NHS-SS-Biotin (Thermo Scientific, Waltham, MA) on ice for 30 min. Cells were washed with PBS, lysed by using lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl,
1% TritonX-100), and then incubated for indicated time at 37°C. Biotinylated proteins were precipitated with streptavidin beads and then subjected to western blot analysis.

Statistics

Results are expressed as the mean ± standard deviation of the mean (S.D.) or standard error of the mean (S.E). Differences between values were statistically analyzed using a Student’s t-test or one-way ANOVA with Bonferroni post-hoc tests (GraphPad Prism 6.0, GraphPad Software, San Diego, CA, USA). A P value < 0.05 was considered statistically significant.
Results

High expression of LOXL2 mRNA in ccRCC specimens

To obtain the mRNA expression signature of ccRCC, we performed an exon array analysis using four ccRCC specimens. Because of its novelty and importance in the cancer biology of ccRCC, LOXL2, which showed a marked increase in the ccRCC tissues compared to the adjacent normal renal tissues (data not shown), was selected for further study. We first conducted quantitative real-time PCR (qPCR) analysis of LOXL2 mRNA in 31 matched-pair ccRCC samples. As shown in Figure 1A, ccRCC tissues significantly expressed more LOXL2 mRNA compared to the matched adjacent normal renal tissues, with an approximately 4-fold increase. Next, the relationship between LOXL2 mRNA levels and the histopathological findings in 121 ccRCC tissue samples were analyzed. LOXL2 mRNA expression significantly correlated with tumor (T) stage (Fig. 1B) and pathological stage (Fig. 1C), but not with the pathological grades (Fig. 1D). These results suggest a relationship between LOXL2 mRNA expression and ccRCC progression.
LOXL2 siRNA knockdown suppresses stress fiber and focal adhesion complex formation in 786-O cells.

To investigate LOXL2 function in ccRCC cells, we evaluated LOXL2 mRNA expression levels in four ccRCC cell lines. Among the ccRCC cell lines, the highest LOXL2 mRNA expression was observed in 786-O cells (Fig. 2A), and therefore, we analyzed LOXL2 function in 786-O cells using an RNA interference method. To assess the knockdown efficiency of LOXL2 siRNAs on its endogenous expression, qPCR and western blot analysis were conducted. As shown in Figure 2B, LOXL2 expression was sufficiently reduced at both the mRNA and protein levels. During the experiment, morphological changes of the LOXL2-knockdowned 786-O cells were observed, and therefore, the knockdown cells were stained with anti-β-actin antibody. LOXL2 siRNAs knockdown markedly reduced the cell extensibility and stress fiber formation, compared to the control siRNA (Fig. 2C and D). Stress fibers are connected to focal adhesion complexes, consisting of integrins and focal adhesion kinase (FAK) (14). Therefore, we investigated the effect of LOXL2 knockdown on focal adhesion formation by staining
with anti-FAK antibody and observed an unusual diffuse FAK localization (Fig. 2D).

Moreover, western blot analysis showed that the level of Tyr$^{576}$-phosphorylated FAK, indicating activated state of FAK (15), was also decreased upon LOXL2 knockdown (Fig. 2E). These results suggest that LOXL2 regulates the formation of stress fibers and focal adhesion complexes in 786-O cells.

LOXL2 upregulates 786-O cell proliferation, migration, and invasion

Because LOXL2 knockdown downregulated FAK activation, which is a key upstream regulator of vast signal transduction (16), we examined the effect of LOXL2 knockdown on additional 786-O cell phenotypes. As shown in Figures 3A, B, and C, the examined LOXL2 siRNAs markedly inhibited 786-O cell proliferation, migration, and invasion. These results indicated that LOXL2 plays an important role in the proliferation, migration, and invasion of 786-O cells.

LOXL2 regulates integrin $\alpha_5$ expression in 786-O cells

Since LOXL2 knockdown reduced focal adhesion formation, we then focused on the
integrin family, a family of focal adhesion core proteins that function as crucial promoters of various cancers (8). Our exon array data showed that integrin α5 expression was most upregulated among the integrin family molecules in all 4 ccRCC tissues compared to adjacent normal renal tissues (Supplementary Fig. 1). We confirmed integrin α5 expression levels by qPCR. Interestingly, correlation (r = 0.60) was shown between LOXL2 and integrin α5 mRNA levels in ccRCC specimens (Fig. 4A). Next, we investigated the effect of LOXL2 knockdown on integrin α5 expression. LOXL2 siRNAs knockdown decreased integrin α5 mRNA expression (Fig. 4B) and markedly reduced integrin α5 protein levels in 786-O cells (Fig. 4C). These results suggest that the phenotypes of LOXL2 knockdown 786-O cells may be manifested through its effects on integrin α5 expression. To test this hypothesis, we transfected integrin α5 siRNA into 786-O cells and examined the phenotypes. As shown in Figures 4D and E, integrin α5 knockdown reduced both stress fibers and focal adhesion formation, which were similar phenotypes to those of LOXL2 knockdown, as shown in Figure 2D. Although integrin α5 knockdown had no effect on proliferation of 786-O cells, cell migration was significantly reduced (Fig. 4F and G). In addition to the
LOXL2 gene, our exon array analysis also showed high LOX gene expression in the four ccRCC clinical samples (Supplementary Fig. 3). To investigate whether LOX also regulates integrin α5 expression and cell migration, we transfected LOX siRNA into Caki1 cells and examined the resulting phenotypes. LOX knockdown had no significant effect on either integrin α5 expression or cell migration ability, but it did suppress focal adhesion formation (Supplementary Fig. 4). These results indicate that LOXL2, but not LOX, maintains the cytoskeleton and promotes migration of ccRCC cells by regulating integrin α5 expression.

**LOXL2 induces 786-O cell adhesion to fibronectin via integrin α5β1 expression**

Integrin α5 associates with integrin β1 to form a heterodimeric receptor (17). Therefore, we examined integrin β1 mRNA and protein expression levels. However, LOXL2 knockdown had no effect on integrin β1 mRNA expression (Fig. 5A). In western blot analysis, two bands of integrin β1, a pre-mature (lower band) and a mature (upper band) form, were detected. Interestingly, mature integrin β1 expression was markedly suppressed upon LOXL2 silencing, but the pre-mature form was unaffected.
(Fig. 5B). On the other hand, LOXL2 overexpression increased the protein expression of integrin α5 as well as both forms of integrin β1 in HEK293T cells (Fig. 5C). From these results, it was presumed that LOXL2 expression was associated with the function of integrin α5/β1 in ccRCC cells. Since the integrin α5/β1 heterodimer specifically binds to fibronectin (18), we performed a fibronectin adhesion assay using 786-O cells to examine the effects of LOXL2 and integrin α5 siRNAs. Both LOXL2 and integrin α5 siRNA knockdowns significantly reduced the adhesive ability of 786-O cells to a fibronectin-coated plate (Fig. 5D). These results supported our hypothesis that LOXL2 modulates a function of integrin α5/β1 in ccRCC cells.

**LOXL2 knockdown downregulates integrin α5 and β1 proteins in a protease- and proteasome-dependent manner in 786-O cells**

Since LOXL2 knockdown reduced the protein expression of integrin α5 and β1 subunits, we then examined the underlying regulatory mechanism of integrin protein expression. First, we utilized the protease inhibitor leupeptin and the proteasome inhibitor MG132 on integrin α5 and β1 protein expression in the LOXL2 knockdown
786-O cells. As shown in Figures 6A and B, both leupeptin and MG132 treatment resulted in an increase in integrin α5 and β1 proteins, compared to LOXL2 siRNA knockdown alone, in a dose-dependent manner. These results suggest that the decreased LOXL2 expression led to degradation of integrin α5 and β1 proteins via the protease and proteasome systems in 786-O cells. Although the precise mechanisms remain largely unknown, internalized integrins α5β1 are either returned to the cell membrane or routed to lysosomes for degradation, and binding to fibronectin regulates the decision to recycle or degrade internalized integrin α5β1 by ubiquitination (19). To clarify if LOXL2 affects integrin α5β1 recycling, we then conducted a cell membrane biotinylation assay. As shown in Figure 6C, LOXL2 knockdown did not affect integrin α5 protein levels in the cell membrane fraction. Moreover, although LOXL2 knockdown reduced integrin α5 and β1 protein expression in the whole cell lysate of 786-O cells, protein levels in the cell membrane fraction were comparable between LOXL2 knockdown and control cells (Fig. 6D). These results suggest that LOXL2 regulates the expression of integrin α5 and β1 proteins not via recycling system, but via protease- and proteasome-dependent systems in 786-O cells.
The relationship between LOXL2 expression and integrin α5β1 expression in ccRCC clinical samples

To examine the expression of LOXL2, integrin α5, and integrin β1 in ccRCC, clinical ccRCC tumor samples were lysed and blotted with anti-LOXL2, anti-integrin α5, and anti-integrin β1 antibodies. Compared with normal human renal epithelial cell lysates, integrin β1 expression protein was comparable, but both LOXL2 and integrin α5 protein expression was elevated in ccRCC samples (Fig. 7).
Discussion

To develop a novel therapeutic drug for ccRCC, it is crucial to clarify the underlying molecular mechanisms involved in the development and progression of ccRCC. In the present study, we showed that LOXL2 was highly expressed in ccRCC clinical samples and induced proliferation, migration, and invasion partly through the suppression of integrin α5β1 degradation through a protease and proteasome-dependent system in ccRCC cells.

LOXL2 promotes EMT by reducing E-cadherin expression via the upregulation of snail expression, which leads to cell shape extension and the acceleration of invasion activity (3, 5). Although we confirmed the reduced snail expression upon LOXL2 knockdown, its effects on E-cadherin and N-cadherin expression were not observed in 786-O cells (Supplementary Fig. 2). On the other hand, we found that LOXL2 knockdown suppressed focal adhesion complex formation and reduced FAK phosphorylation (Fig. 2C, D, and E). FAK critically regulates cell migration and invasion in several cancers by regulating focal adhesion complex formation and matrix
metalloproteinase expression (20). These results suggest that the highly expressed LOXL2 promotes migration and invasion of ccRCC cells not via EMT progression but via regulation of stress fiber and focal adhesion complex formation.

LOXL2 is a member of the LOX family, which comprises the 5 members LOX and LOXL1–4. The LOX family contains a highly conserved lysyl oxidase domain, which catalyzes the covalent crosslinking of component side chains of collagen and elastin (3). LOX knockdown in Caki1 cells, however, did not significantly affect either the integrin α5 expression or migration ability, but it did suppress focal adhesion formation (Supplementary Fig. 4). LOXL2 contains not only the lysyl oxidase domain, but also 4 scavenger receptor cysteine-rich (SRCR) domains (5), which are generally found in secreted or cell surface-bound proteins, and is involved in cell adhesion and cell signaling by mediating protein-protein interactions, as reported in CD6 and ALCAM (21-23). Lugassy et al. have demonstrated that the SRCR domain, but not the enzyme activity via the lysyl oxidase domain, is required for the LOXL2-induced inhibition of keratinocyte differentiation (24). Therefore, LOXL2-mediated regulation of integrin α5 expression in ccRCC cells might happen irrespective of the lysyl oxidase
domain and may be a function of the SRCR domain.

The upregulated integrin α5 mRNA expression in ccRCC specimens correlated with LOLX2 mRNA expression (Fig. 4A). However, LOLX2 knockdown significantly downregulated integrin α5 expression at the protein level but not the mRNA level (Fig. 4B and C). TGF-β signaling could explain these contradictory data. The TGF-β signaling pathway is known to be involved in cell proliferation, differentiation, apoptosis, and EMT in various cancer cell types (25). In ccRCC cells, although the precise regulatory mechanism remains largely unknown, TGF-β expression correlates with pathological stages and grades, and TGF-β promotes the establishment of bone metastasis in RCC (26, 27). Moreover, the TGF-β signaling pathway has been reported to upregulate both integrin α5 (28) and LOXL2 expression (29). Our preliminary data also showed that TGF-β treatment increased the mRNA expression levels of both LOXL2 and integrin α5 in 786-O cells (Supplementary Fig. 5). These results suggested that LOXL2 and integrin α5 are induced by TGF-β at the transcriptional level, and LOXL2 is involved in integrin α5 protein stabilization in ccRCC cells.

Integrin turnover has been implicated in trafficking, including internalization,
recycling, and degradation. Integrins internalized by endocytosis are either returned to the cell membrane or routed for degradation via lysosomes and/or the proteasome. Sorting nexin-17 (SNX17) controls recycling or degradation of integrin β1 by ubiquitination, and SNX17 knockdown downregulates only the mature form of integrin β1 (30). Our data show that LOXL2 knockdown downregulates only the mature form of integrin β1. Therefore, LOXL2 might regulate integrin β1 protein expression via SNX17 in ccRCC cells.

In response to fibronectin binding, both integrins α5 and β1 expressed on cell membrane undergo ubiquitination and subsequent proteasome-dependent degradation via endosomal sorting complex required for transport (ESCRT) machinery (19). Our data showed that LOXL2 knockdown induced protease- and proteasome-dependent degradation of integrin α5 and β1 proteins, without affecting the half-life of their membrane bound forms in 786-O cells (Fig. 6A, B, and D). The same results were also found in the fibronectin binding experiment (Fig. 6C). Although the precise mechanisms are largely unknown, Sayeed et al. showed that insulin-like growth factor receptor stabilizes integrin α5β1 by protecting it from proteasomal degradation in a
fibronectin-independent manner (31). Therefore, LOXL2 could intracellularly regulate integrin α5 and β1 protein degradation regardless of the fibronectin-dependent degradation system in ccRCC. Further studies are required to resolve this issue.

Although the function of integrin α5 on cancer progression is controversial (32-35), integrin α5 has been shown to contribute to the acquisition of resistance to apoptosis, angiogenesis, invasion, and metastasis, and it has thus attracted attention as a target for ccRCC therapy (36, 37). Interestingly, Mierke et al. have reported that in a 3D collagen invasion assay, integrin α5 accelerates invasive activity by regulating the cell adhesiveness and contractile forces in 786-O cells, instead of regulating the secretion of proteolytic enzyme (38). Moreover, integrin α5 binds to c-MET fibronectin-independently and promotes the invasion of ovarian cancer cells (39). Therefore, LOXL2 might regulate cytoskeletal maintenance and invasion of ccRCC cells via integrin α5.

In conclusion, our present study shows that intracellular but not secreted LOLX2 transduces enhanced focal adhesion signaling through the stabilization of integrin α5β1 expression in ccRCC cells. The high expression of LOXL2 promotes migration and
invasion via this novel mechanism, causing the progression of ccRCC.
Acknowledgements

The study was supported by a Grant-in-Aid for Scientific Research (25670025 and 25893113) and by Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by Project MEET, Osaka University Graduate School of Medicine.
References


6. Amparo C, Patricia G S, Gema Moreno-B. LOXL2 in epithelial cell plasticity and


LT. Integrin expression profiling identifies integrin α5 and β1 as prognostic factors in early stage non-small cell lung cancer. Molecular Cancer 2010;9:152.


Table 1

ccRCC clinical samples used for exon array analysis

<table>
<thead>
<tr>
<th></th>
<th>Patient A</th>
<th>Patient B</th>
<th>Patient C</th>
<th>Patient D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>67</td>
<td>86</td>
<td>60</td>
<td>66</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>T1</td>
<td>T3</td>
<td>T3</td>
<td>T4</td>
</tr>
<tr>
<td>Pathological grade</td>
<td>G2</td>
<td>G2</td>
<td>G2</td>
<td>G3</td>
</tr>
<tr>
<td>TNM classification</td>
<td>I</td>
<td>III</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Matched-pair sample</td>
<td>Tumor only sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------------</td>
<td>-------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>65</td>
<td>65.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>34-86</td>
<td>34-82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>19</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>3</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>8</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>12</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>15</td>
<td>57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>4</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. LOXL2 mRNA expression was upregulated in ccRCC samples.

LOXL2 mRNA expression was examined in 31 matched-pair ccRCC clinical samples (A) and 121 ccRCC clinical samples (B-D) by qPCR. Data are the relative expression normalized to GAPDH mRNA. The relative expression of LOXL2 mRNA was compared by T stages (B), pathological stages (C), and pathological grades (D). Values are means ± S.D., *P < 0.05, ***P < 0.001.

Figure 2. LOXL2 knockdown reduced stress fiber and focal adhesion formation in 786-O cells.

A, LOXL2 mRNA expression in four ccRCC cell lines was examined by qPCR. Values are means ± S.D. of three independent experiments. B, 786-O cells were transfected with control (CNT) siRNA or two LOXL2 siRNAs for 48 h. LOXL2 mRNA and protein expression levels were examined by qPCR and by western blotting with anti-LOXL2 and anti-β-actin antibodies, respectively. For qPCR analysis, values are mean ± S.D. of
three independent experiments. For western blot analysis, representative pictures of three independent experiments are shown. C and D, 786-O cells were transfected with control (CNT) siRNA or two LOXL2 siRNAs for 48 h, and then immunofluorescence staining of the cells was performed with the anti-β-actin (C) and with the anti-β-actin and anti-FAK antibodies (D). Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI). Representative pictures of three independent experiments are shown. White bars indicate 50 μm for (C) and 10 μm for (D). E, Protein lysates from 786-O cells transfected with control (CNT) siRNA or LOXL2 siRNA for 48 h were subjected to western blot analysis with anti-phospho-FAK (Tyr576), anti-FAK, and anti-β-actin antibodies. The blots shown are representative of three independent experiments.

**Figure 3.** LOXL2 knockdown suppressed proliferation, migration, and invasion of 786-O cells.

786-O cells were transfected with control (CNT) siRNA or LOXL2 siRNA for 24 h. A, The transfected cells were inoculated in an E-plate, and the cell proliferation was
monitored in real-time for 72 h using the xCELLigence system. Values expressed as cell
index are the means ± S.D. of duplicated experiments. B, The transfected cells were
replated at 90% confluence, and cell migration was measured 12 h after a wound was
formed by scraping. Values are means ± S.D. of three independent experiments. C, The
transfected cell suspension was added to the upper chamber of a matrigel-coated
CIM-plate. The lower chamber was filled with medium and then cultured for 18 h in the
xCELLigence system. Values expressed as cell index are means ± S.D. of duplicated
experiments.

**Figure 4. LOXL2 knockdown downregulated integrin α5 expression in 786-O cells.**

A, The expression of LOXL2 and integrin α5 mRNAs was examined in 31 matched-pair
samples of ccRCC by qPCR, and the relationship was evaluated. 786-O cells were
transfected with control (CNT) siRNA or two LOXL2 siRNAs for 48 h. B, Integrin α5
mRNA expression was examined by qPCR and normalized to GAPDH mRNA
expression. C, Integrin α5 protein expression was analyzed by western blot with an
anti-integrin α5 antibody and normalized to β-actin protein. The blots shown are
representative of three independent experiments. D, Protein lysates from 786-O cells transfected with control (CNT) siRNA or two integrin α5 siRNAs for 48 h were subjected to western blot analysis with integrin α5 and anti-β-actin antibodies. The blots are representative of three independent experiments. E, 786-O cells were transfected with control (CNT) siRNA or two integrin α5 siRNAs for 48 h, and immunofluorescence staining was performed with anti-β-actin and anti-FAK antibodies. Nuclear staining was performed using 4′,6-diamidino-2-phenylindole (DAPI). Representative pictures of 3 independent experiments are shown. The white bar indicates 10 μm. F, 786-O cells were transfected with control (CNT) siRNA or two integrin α5 siRNAs for 24 h, and then, cell proliferation was examined by WST-1 assay. Values are means ± S.D. of three independent experiments. G, The transfected cells were reseeded at 90% confluence, and cell migration was measured 12 h after a wound was formed by scraping. Values are means ± S.D. of three independent experiments. *P < 0.05 vs. control siRNA.

Figure 5. LOXL2 regulated adhesion of 786-O cells to fibronectin via integrin

44
**α5/β1 expression**

786-O cells were transfected with control (CNT) siRNA or two LOXL2 siRNAs for 48 h. Integrin β1 mRNA and protein expression levels were examined by qPCR and by western blotting with anti-integrin β1 antibody, respectively. A, For qPCR analysis, values were normalized to GAPDH mRNA expression and are the means ± S.D. of three independent experiments. B, For western blot analysis, representative pictures of three independent experiments are shown. Values, normalized to β-actin protein, are means ± S.E. of three independent experiments. C, HEK 293T cells were transfected with mock or LOXL2 expression vectors for 72 h, and protein samples were collected and subjected to western blot analysis with anti-LOXL2, anti-integrin α5, and anti-integrin β1 antibodies. Representative pictures of three independent experiments are shown. D, 786-O cells were transfected with control (CNT) siRNA, LOXL2 siRNAs, or integrin α5 siRNAs for 48 h and recultured on fibronectin-coated wells for 30 min. After PBS washing, adherent cells were stained with calcein-AM, and the fluorescence intensity was measured. Values are means ± S.D. of five independent experiments. *P < 0.05 compared to control siRNA.
**Figure 6. LOXL2 suppressed integrin α5 and integrin β1 protein degradation in 786-O cells.**

786-O cells were transfected with control (CNT) siRNA or LOXL2 siRNA and treated with indicated concentrations of leupeptin for 1 h (A) or MG132 for 12 h (B). The cell lysates were subjected to western blot analysis with anti-integrin α5 and anti-integrin β1 antibodies. Representative pictures of three independent experiments are shown. C, 786-O cells were transfected with control (CNT) siRNA or LOXL2 siRNA and cultured on a fibronectin-coated plate for 24 h. The cell membrane proteins were biotinylated for 0, 3, and 6 h, harvested by precipitation with streptavidin beads, and then subjected to western blot analysis with anti-integrin α5 and anti-β-actin antibodies. Representative pictures of three independent experiments are shown. D, The cell membranes of 786-O cells transfected with control (CNT) siRNA or LOXL2 siRNA for 24 h were biotinylated. After cell lysis, biotinylated proteins were harvested by precipitation with streptavidin beads and then subjected to western blot analysis with anti-integrin α5, anti-integrin β1, and anti-β-actin antibodies. Values, normalized to integrin α5 and anti-integrin β1 protein levels in whole cell lysate, are means ± S.E. of three
independent experiments.

Figure 7. High expression of LOXL2 and integrin α5 was detected in ccRCC clinical samples.

The expression levels of LOXL2, integrin α5, integrin β1, and β-actin in lysates of ccRCC tumor clinical samples were examined by western blot analysis with their respective antibodies. 11 ccRCC samples and one normal renal epithelial cells were examined. G1, G2, and G3 are pathological grades. T1, T2, and T3 are the classifications of tumor by the size and extent.
Figure 3

A

B

C

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 7

<table>
<thead>
<tr>
<th></th>
<th>LOXL2</th>
<th>Integrin α5</th>
<th>Integrin β1</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human renal epithelial cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>TI</td>
<td>T1</td>
<td>TI</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>T1</td>
<td>T1</td>
<td>T1</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>T1</td>
<td>T1</td>
<td>T1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T3</td>
</tr>
</tbody>
</table>
Molecular Cancer Research

LOXL2 Status Correlates with Tumor Stage and Regulates Integrin Levels to Promote Tumor Progression in ccRCC

Hiroaki Hase, Kentaro Jingushi, Yuko Ueda, et al.

Mol Cancer Res  Published OnlineFirst August 4, 2014.

Updated version
Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-14-0233

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.