

Lysosome-associated membrane protein 2 (LAMP-2) expression induced by miR-194-5p downregulation contributes to sunitinib resistance in human renal cell carcinoma cells

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Abstract. Sunitinib is a tyrosine kinase inhibitor that is used as the primary treatment in metastatic renal cell carcinoma (RCC). The main difficulty associated with its use is the development of drug resistance. In the present study, ACHN cells, a human renal cell carcinoma cell line, were used to establish sunitinib-resistant (SR) cells. Microarray analysis and reverse transcription-quantitative polymerase chain reaction revealed that miR-194-5p expression was significantly decreased in SR-ACHN cells when compared with that observed in ACHN cells ($P < 0.05$). Transfection of miR-194-5p, though not with negative control miR, in SR-ACHN cells could significantly inhibit cell proliferation following sunitinib treatment (2.5–40 μM ; $P < 0.05$). Western blotting demonstrated that the expression of lysosome-associated membrane protein-2 (LAMP-2), which attenuates the anti-proliferative effect of sunitinib, was significantly higher in SR-ACHN than in ACHN cells ($P < 0.01$). In addition, LAMP-2 expression was suppressed by miR-194-5p transfection in SR-ACHN cells. These data suggested that miR-194-5p downregulation may be associated with sunitinib resistance via the induction of LAMP-2 expression in human RCC.

Introduction

The number of renal cell carcinoma (RCC) patients is increasing worldwide. Approximately 20% of RCC patients present with advanced stage disease at the time of diagnosis, and in patients with localized RCC, nearly 30% will experience disease

recurrence after tumor resection (1,2). Targeted therapy using tyrosine kinase inhibitors (TKIs) is used as a treatment for metastatic renal cell carcinoma (mRCC). TKIs inhibit multiple receptor tyrosine kinases needed for the activation of intracellular signaling pathways controlling cell proliferation, survival, or angiogenesis. Among TKIs, sunitinib, an orally available TKI, is the most commonly used molecular-targeting agent as first-line therapy for mRCC (3). In the sunitinib registration trial, half of the treated patients with a favorable or intermediate risk score based on Memorial Sloan-Kettering Cancer Center criteria achieved an objective response, resulting in a median progression-free survival (PFS) of 11 months (4,5). However, the clinical benefit of sunitinib in PFS is limited, and the majority of mRCC patients treated with sunitinib ultimately experience disease progression due to the acquisition of resistance. In such cases, progressed patients require further sequential therapy using other TKIs or mTOR inhibitors (mTORIs). In spite of treatment of the second therapy, the median PFS was short; 4.8 months with axitinib, 3.4 months with sorafenib, 4.3 months with temsirolimus, 7.5 months with pazopanib, and 4.0 months with everolimus (6–9). Therefore, we thought that it should be necessary to lengthen PFS in first-line therapy of sunitinib. Identifying pathways responsible for intrinsic or acquired resistance could provide novel directions to develop therapies that block resistance pathways.

Intracellular drug accumulation accompanied by increased lysosomal storage is elevated in sunitinib-resistant cells (10). Moreover, the lysosomal capacity is enhanced by upregulating lysosome-associated membrane protein-1 and -2 (LAMP1/2) expression (11). Based on these data, identifying mechanisms responsible for intrinsic or acquired sunitinib resistance involving LAMP1/2 could provide novel directions to develop therapies that block resistance pathways.

microRNAs (miRNAs) are noncoding single-stranded small RNAs (~ 22 nucleotides) that regulate posttranscriptional gene expression. miRNA levels are altered in many human diseases including cancer (12). miRNAs play an important role as regulators of gene expression in tumorigenesis, tumor progression, drug resistance, and metastasis (13,14). Thus, we previously generated a sunitinib-resistant RCC cell line,

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SR-ACHN (sunitinib-resistant ACHN), by continuous treatment with sunitinib to detect candidate miRNAs implicated in the regulation of sunitinib resistance (15). The aim of this study was to identify miRNAs that suppressed sunitinib resistance via LAMP2 expression using ACHN and SR-ACHN cells.

Materials and methods

Cell lines. A human RCC cell line, ACHN, was purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific Inc.). Sunitinib-resistant ACHN (SR-ACHN) cells were generated as previously described (15). SR-ACHN cells were maintained in the same medium containing 10 μ M sunitinib (Sigma Aldrich, St. Louis, MO, USA).

Clinical samples. Twelve samples of advanced RCC, which had lymph node metastasis or distant metastasis, were obtained from Tottori University Hospital. All the materials were obtained with informed consent, and the procedures were approved by The Ethics Committee of Tottori University (Tottori, Japan; approval number: 1558). Tissue samples were obtained from tumor tissues and matched normal tissues from the same kidney specimen in RCC patients. Normal tissues were far from tumor clearly macroscopically.

Total RNA extraction and microarray. Total RNA from ACHN and SR-ACHN cells was extracted using the miR-Vana™ miRNA isolation kit (Thermo Fisher Scientific Inc.) following the manufacturer's protocol. Total RNA quality control for quantity and purity was determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc.). The RNA samples were stored at -80°C until the reverse transcription (RT) reaction. For microarray analysis, total RNA was labeled using a 3D-Gene miRNA labeling kit (Toray Industries; Kamakura, Japan). Labeled RNA was hybridized to 3D-Gene human miRNA V21 chips (Toray Industries).

Transfection with synthetic miRNAs into SR-ACHN cells and proliferation assay. Synthetic human miRNAs (hsa-miRs) [hsa-miR-194-5p and negative control (NC); Ambion] were transfected into SR-ACHN cells at 60 nmol/l (final concentration) per 3×10^6 cell/well in a 10-cm dish, using DharmaFECT (GE Healthcare, Pittsburgh, PA). After 24 h of incubation, cells were harvested and reseeded into a 96-well plate. miR transfected cells were plated at 5×10^3 cells/well in a 96-well plate. After 24 h, sunitinib was added at different concentrations and proliferation after 72 h was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol.

RNA extraction and quantitative real-time PCR of miRNAs. Total RNA from cell lines and clinical samples was extracted using a miR-Vana™ miRNA Isolation Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. miR-194-5p-specific complementary DNA was generated from 20 ng total RNA, using the TaqMan MicroRNA RT kit (Applied Biosystems, Foster City, CA) and the miR-194-5p-specific

RT-primer from the TaqMan Micro RNA Assay (Applied Biosystems). miR-194-5p levels were also measured using the miR-194-5p-specific probe included with the TaqMan Micro RNA Assay on an ABI 7900HT System and SDS software (Applied Biosystems).

Western blotting. The cells were lysed with cell lysis buffer {1 mmol/l Na_3VO_4 , 100 mmol/l phenylmethylsulfonyl fluoride (PMSF), 500 mmol/l NaF, 500 mmol/l ethylenediamine-tetraacetic acid (EDTA), 10% NP-40, 2 mg/ml aprotinin, 2 mg/ml leupatin, 1 mol/l Tris pH 7.6, 5 mol/l NaCl and distilled water}. Protein concentrations were determined by the Micro BCA protein assay (Thermo Fisher Scientific Inc.). Samples containing 20 μ g protein underwent electrophoresis on 10% SDS polyacrylamide gels and were subsequently transferred to PVDF membranes. The membranes were blotted with a mouse monoclonal antibody against LAMP-2 (1:250; ab119124; Abcam, Cambridge, MA), or with a monoclonal antibody against β -actin (1:2,000; AC-15; Sigma, Aldrich).

Signals were visualized using an enhanced chemiluminescence system (ECL Detection System; Amersham Pharmacia Biotech, Piscataway, NJ).

Immunohistochemistry. Clinical tissues were fixed in 10% formalin and embedded in paraffin. Sections 3- μ m thick were examined by immunohistochemistry. The sections were deparaffinized and antigens were retrieved using an autoclave in 10 mmol/l citrate buffer (pH 6.0) at 121°C for 10 min. Endogenous peroxidase activity was blocked by immersing the slides in 0.6% hydrogen peroxide in methanol for 20 min. The sections were immunostained using a Histofine rabbit stain kit (Nichirei, Tokyo, Japan). The primary antibody was a mouse monoclonal antibody against LAMP-2 (1:10; ab25631; Abcam) followed by incubation with secondary antibodies. Immunoreactions were visualized with diaminobenzidine and the sections were counterstained with hematoxylin.

Statistical analysis. Statistical significance was determined by the two-tailed unpaired Student's t-test and using the Pearson correlation coefficient. Differences were considered to be statistically significant when $P < 0.05$.

Results

miRNA microarray analysis and validation of the array data by real-time RT-qPCR. A pair of cell lines (ACHN and SR-ACHN) was used to identify miRNA candidates involved in regulating sunitinib resistance, based on the premise that sunitinib resistance-related miRNAs are identifiable by comparing the miRNA expression patterns in these cells. miRNA microarray analysis was performed by comparing ACHN and SR-ACHN cells to evaluate the miRNA profiles of each cell type. The expression levels of many miRNAs were different between the two cell lines. Fifteen miRNAs were significantly upregulated over 8-fold (sunitinib-resistant miRNAs) whereas thirty-one miRNAs, including miR-194-5p, were significantly down-regulated over 4-fold in SR-ACHN compared with that in ACHN cells (Table I).

On the basis of the microarray results, we further examined the expression level of miR-194-5p by real-time RT-qPCR. We

Table I. microRNAs in sunitinib-resistant ACHN cells increase 8-fold and decrease 4-fold when compared with ACHN cells.

Name	Ratio of ACHN/SR-ACHN cells
A, Upregulated miRNA	
miR-575	16.71
miR-4459	15.30
miR-6088	15.05
miR-4430	13.35
miR-642b-3p	12.40
miR-4294	11.46
miR-6808-5p	11.13
miR-6769b-5p	10.24
miR-675-5p	9.37
miR-6076	9.10
miR-671-5p	8.68
miR-6501-3p	8.47
miR-4651	8.32
miR-4467	8.15
miR-4433b-3p	8.05
B, Downregulated miRNA	
miR-7-5p	0.05
miR-29b-1-5p	0.06
miR-155-5p	0.10
miR-4521	0.12
miR-29a-5p	0.13
miR-652-3p	0.13
miR-192-5p	0.14
miR-194-5p	0.14
miR-16-2-3p	0.14
miR-215-5p	0.16
miR-222-5p	0.16
miR-518b	0.17
miR-3194-3p	0.17
miR-21-3p	0.18
miR-18a-5p	0.18
miR-376c-3p	0.18
miR-20a-3p	0.20
miR-495-3p	0.20
miR-3200-3p	0.20
miR-3175	0.21
miR-18b-5p	0.22
miR-20b-5p	0.23
miR-431-3p	0.23
miR-454-3p	0.24
miR-130b-3p	0.24
miR-590-5p	0.24
miR-301a-3p	0.24
miR-106a-5p	0.24
miR-4284	0.25
miR-4259	0.25

miR, microRNA; SR, sunitinib-resistant.

Table II. Patient characteristics (n=12).

Characteristic	Number of patients (n)
Age (years)	
Mean	60
Range	26-70
Sex	
Male	6
Female	6
Histopathology	
Clear cell	6
Papillary	2
Chromophobe	2
Spindle	2
Pathological stage	
pT3	8
pT2	1
pT1	3
Lymph nodes metastasis	5
Distant metastasis	10
Grade	
G3	9
G2	3

selected miR-194-5p based on a previous study, which reported that higher miR-194 expression correlated with significantly longer disease-free survival and overall survival compared to those with lower expression in patients with RCC (16). RNA pools from the same RNA samples used for the microarray experiments were prepared. We used *RNU6B* as a reference gene for normalization of the miRNA data. The CT values of miR-194-5p were significantly decreased in SR-ACHN cells compared with that in ACHN cells ($P < 0.01$) (Fig. 1A). The PCR result was consistent with the microarray data.

miRNA mimic oligonucleotide transfer restores sunitinib resistance in ACHN cell lines. The IC₅₀ concentration of sunitinib for ACHN and SR-ACHN cells was 10 and 21 μM , respectively, showing that SR-ACHN cells exhibited significantly higher resistance to sunitinib treatment compared with ACHN cells, as previously reported by Yamaguchi *et al* (15). When SR-ACHN cells transfected with miR-194-5p or negative control miR were treated with sunitinib, the number of live cells was significantly decreased in miR-194-5p-transfected cells than in negative control miR-transfected SR-ACHN cells at a sunitinib concentration range of 2.5 to 40 μM (Fig. 1B).

Detection and identification of miR-194-5p target genes. To elucidate sunitinib resistance-related miR-194-5p target genes in SR-ACHN cells, candidate target genes were selected using miRDB 5.0. Out of the many miR-194-5p target genes, we focused on *LAMP2*, which is known to contribute to sunitinib resistance in renal cell cancer cells (17). In fact, western blot analysis revealed that *LAMP2* protein expression was markedly

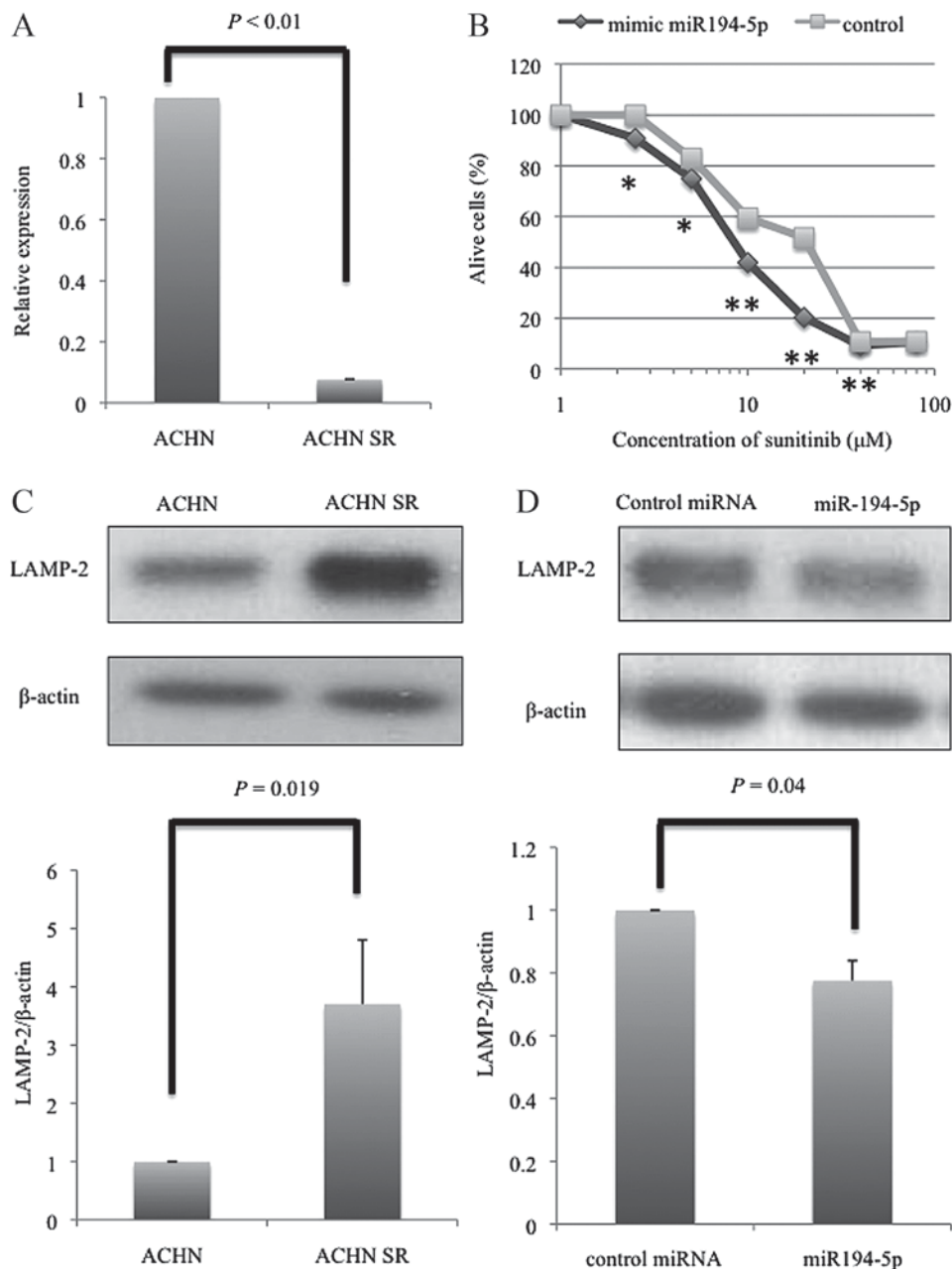


Figure 1. (A) Quantitative miRNA levels in ACHN cells compared with that observed in SR-ACHN cells. miR-194-5p levels were significantly decreased in SR-ACHN cells. (B) Effects of *in vitro* sunitinib treatment on cell growth. SR-ACHN cells transfected with mimic miR-194-5p exhibited significantly lower resistance to sunitinib treatment when compared with SR-ACHN cells transfected with control miRNA. * $P < 0.05$ and ** $P < 0.01$ vs. control. (C) Western blot analysis of LAMP-2 in sunitinib-sensitive and SR-ACHN cells. LAMP-2 expression in SR-ACHN cells was higher than that observed in ACHN cells. (D) LAMP-2 was downregulated by miR-194-5p. Western blot analyses of LAMP-2 expression in SR-ACHN cells 48 h following transfection of miR-194-5p or control miRNA. Relative expression was quantified using ImageJ software, and normalized to β -actin; it is reported as the ratio of the indicated situation to control miRNA. SR, sunitinib-resistant; LAMP2, lysosome associated membrane protein-2; miR/miRNA, microRNA.

higher in SR-ACHN cells than in ACHN cells ($P = 0.019$, Fig. 1C). These data motivated us to examine whether miR-194-5p could suppress the expression of LAMP2 in SR-ACHN cells. As shown in Fig. 1D, the expression of LAMP-2 was significantly decreased by miR-194-5p transfection compared with that by miR-NC transfection ($P = 0.04$).

Expression of miR-194-5p and LAMP-2 in clinical samples. Finally, we evaluated the expression of miR-194-5p in human advanced RCC samples from radical nephrectomies. The characteristics of clinical samples are shown in Table II.

Twelve samples of advanced RCC were analyzed for the expression of miR-194-5p by RT-qPCR. The miR-194-5p expression data were normalized to that of *RNU6B*. Tissue miR-194-5p levels were significantly lower in tumor tissues than in normal tissues ($P = 0.004$, Fig. 2A). LAMP-2 expression was evaluated by immunohistochemistry. As shown in Fig. 2B, LAMP2 immunoreactivity was observed in tumor cell cytoplasm. The percentage of LAMP-2-positive tumor cells was inversely correlated to miR-194-5p expression levels ($r = -0.60$, $t = -2.39$, $P = 0.038$). Although we analyzed the relationship between miR-194-5p or LAMP-2 expression

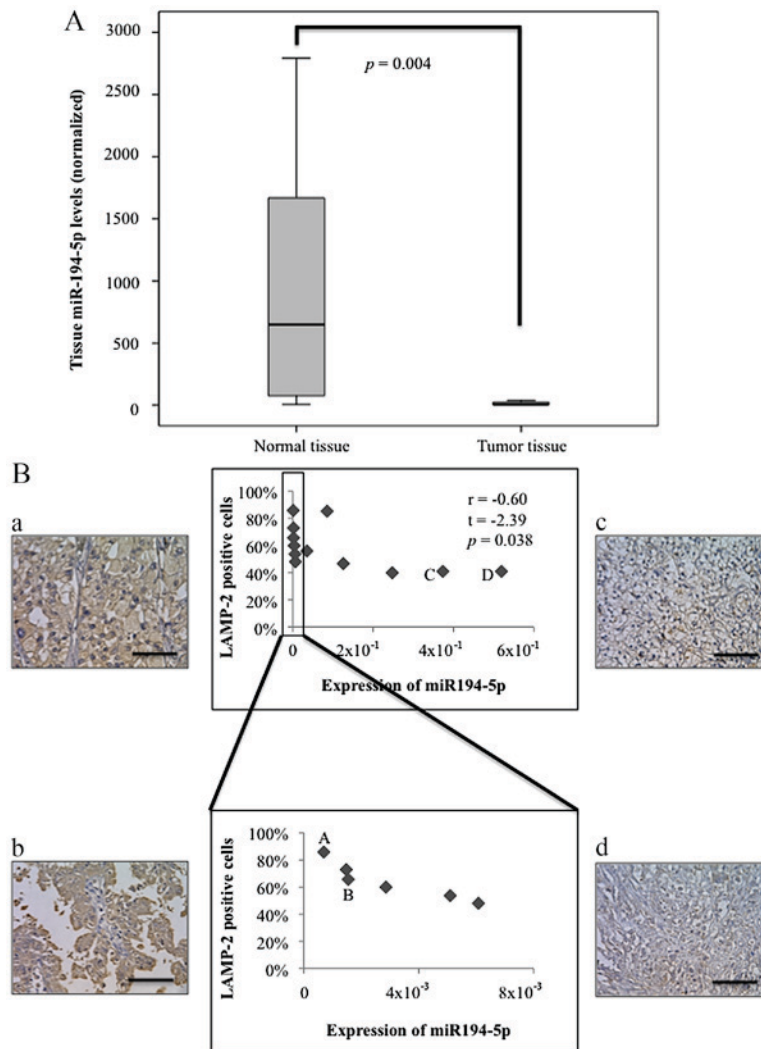


Figure 2. (A) Quantitative miRNA levels in tumor tissues compared with those observed in normal tissues. The miR-194-5p expression level was significantly decreased in tumor tissues ($P=0.004$). (B) LAMP2 immunoreactivity was observed in the tumor cell cytoplasm. The percentage of LAMP-2-positive tumor cells associated with miR-194-5p expression levels are presented ($r=-0.60$, $t=-2.39$, $P=0.038$). Staining images A-D represent the stained cells analyzed to produce the indicated data points (A-D) on the graph. Scale bars, $100 \mu\text{m}$. LAMP2, lysosome associated membrane protein-2; miR, microRNA.

and clinicopathological parameters, there were no significant differences in T stage, lymph node metastasis, and distant metastasis for miR-194-5p (Fig. 3) or LAMP-2 (Fig. 4).

Discussion

Although sunitinib is a TKI indicated as a first-line treatment for mRCC, the clinical benefit of sunitinib in PFS is limited, and the majority of mRCC patients treated with sunitinib ultimately experience disease progression due to the acquisition of resistance (3-5). miRNAs play a crucial role in modulating the sensitivity to chemotherapeutic agents in multiple tumors (18,19), indicating that miRNAs are promising therapeutic targets in cancers. Therefore, identifying miRNAs that could eliminate resistance to sunitinib in RCC cells may help elucidate the mechanism of sunitinib resistance and clinically benefit RCC patients. The present study revealed that restoring miR-194-5p expression in SR-ACHN cells sensitized to sunitinib via down-regulating LAMP2, a possible target of miR-194-5p in RCC cells, increased sunitinib sensitivity. It is strongly suggested that over-expression of LAMP2

by inhibiting miR-194-5p may lead to sunitinib resistance acquisition.

There is only one study showing that miR-194-5p is associated with drug resistance in human cancers. Zhu *et al* reported that miR-194-5p is down-regulated in the cisplatin-resisted human non-small cell lung cancer cell line-A549/DDP and over-expression of miR-194-5p increases cisplatin sensitivity via down-regulating FOXA1, a target of miR-194-5p (20). However, the paper reported that down-regulation of miR-194-5p contributed to drug resistance against cisplatin, but not sunitinib. So far, various miRNAs have been reported to contribute to sunitinib resistance in RCCs (12,21-23). For example, Merhautova *et al* reported that decreased tissue levels of miR-155 and miR-484 are significantly associated with prolonged time to progression in RCC patients treated with sunitinib (21). In addition, Berkers *et al* reported that miR-141 down-regulation-driven epithelial-to-mesenchymal transition (EMT) in clear cell carcinoma is associated with an unfavorable response to sunitinib, indicating that low miR-141 expression results in poor prognosis (22). Goto *et al* reported that miR-101 is markedly suppressed in sunitinib-treated

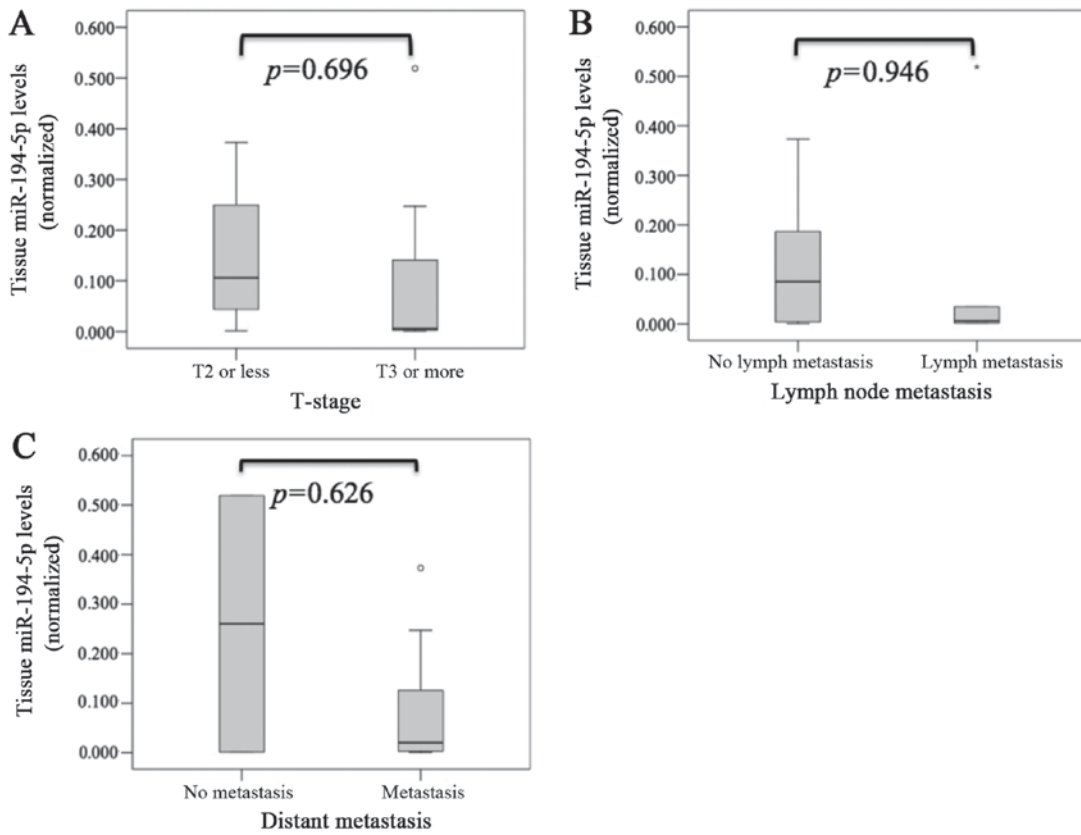


Figure 3. Analysis of the associations between tissue miR-194-5p levels and clinicopathological parameters. There were no significant associations between miR-194-5p tissue expression levels and (A) T stage, (B) existence of lymph node metastasis, or (C) existence of distant metastasis. miR, microRNA.

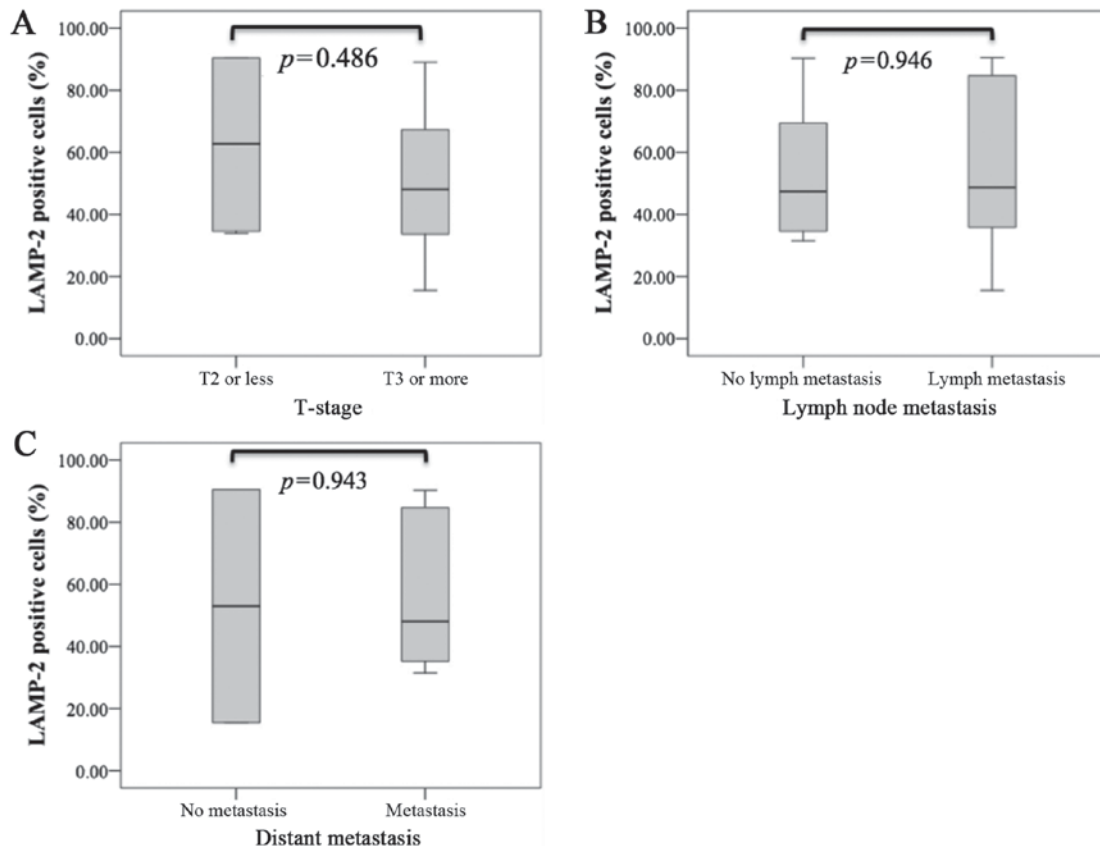


Figure 4. Analysis of the associations between LAMP-2 positive cells and clinicopathological parameters. There were no significant associations between tissue LAMP-2 levels and (A) T stage, (B) existence of lymph node metastasis or (C) existence of distant metastasis. LAMP2, lysosome associated membrane protein-2.

RCC tissues and restoration of miR-101 significantly inhibits migration and invasion in Caki-1 and 786-O cells (23). A comprehensive analysis screened 673 miRNAs in tumor tissues from mRCC patients and revealed that higher miR-942 expression is an independent predictor of inadequate sunitinib efficacy (12). Although such miRNAs related to sunitinib resistance might be useful as prognostic markers, no data was shown regarding whether the altered expression of such miRNAs directly gave rise to sunitinib-resistant RCCs. Our data showed that not only low-expression of miR-194-5p was associated with resistance against sunitinib, but also susceptibility to sunitinib was ameliorated by miR-194-5p restoration in RCC cells via LAMP-2 down-regulation.

LAMP-2 is present at the lysosomal membrane and contributes to lysosome function. In recent studies, the lysosome was associated with the sunitinib resistance mechanism. Giuliano *et al* reported that sequestration of sunitinib in lysosomes and the subsequent inhibition of the autophagy flux participate in sunitinib resistance (24). The incomplete autophagic flux is caused by suppression of the lysosomal protease cathepsin B activity. In addition, Gotink *et al* reported fluorescent microscopy data revealing intracellular sunitinib distribution mainly in acidic lysosomes, which are also significantly increased in sunitinib-resistant renal cancer cells compared to that in parental cells (25). These data indicate that sunitinib resistance is dependent on the lysosomal capacity, which is reflected by the LAMP-2 expression level. Therefore, expansion of sunitinib accumulation in lysosomes may be induced by increasing LAMP-2 expression, contributing to sunitinib resistance in mRCC cells.

We investigated the relationship between miR-194-5p or LAMP-2 expression and T-stage, lymph node metastasis, and distant metastasis, and found there were no significant differences. In the previous report, Lee *et al* reported that miR-194-5p might be used as diagnostic biomarkers in adenocarcinoma in uterine cervix, but there were no significant differences between miR-194-5p and T-stage, lymph node metastasis, and distant metastasis (26). In addition, LAMP-1 might influence local tumor progression rather than the formation of tumor metastasis in pancreatic carcinoma, but no relation was found between LAMP-2 and the tumor stage or lymph node metastasis (27). In lung cancer, Giatromanolaki *et al* reported that LAMP-2 was related to high histology grade, and was not related tumor stage (28). However, many reports described that LAMP2 was related to drug resistance (24,25). These data showed that these two molecules contributed to drug resistance acquisition by sunitinib uptake by lysosomes, and suggested that down-regulation of LAMP-2 by miR-194-5p was independent of cell proliferation, cell death resistance, invasion, and metastasis. Therefore, we thought that clarifying the intracellular signaling transmission pathways regulated by miR-194-5p could identify mechanisms responsible for intrinsic or acquired sunitinib resistance.

In conclusion, we have identified miR-194-5p as a sunitinib-resistant suppressive miRNA that down-regulates LAMP2 in human RCC cells. Targeting miR-194-5p could contribute to a new therapy against sunitinib resistance and improve PFS for patients with mRCC.

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