# Splice variants of lysosome-associated membrane proteins 2A and 2B are involved in sunitinib resistance in human renal cell carcinoma cells

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**Abstract.** Sunitinib, a tyrosine kinase inhibitor, is among the first-line treatments for metastatic or advanced stage renal cell carcinoma (RCC). However, patients with RCC develop resistance to sunitinib. We have previously demonstrated that lysosome-associated membrane protein 2 (LAMP-2), which has three splice variants with different functions (LAMP-2A, LAMP-2B, and LAMP-2C), is involved in RCC. In the present study, we examined which splice variants of LAMP-2 contributed to sunitinib resistance in RCC cells. *In vitro* analysis using ACHN, human RCC cell line, revealed that the $IC_{50}$ of sunitinib was significantly increased by overexpression of LAMP-2A and LAMP-2B, but not LAMP-2C ($P<0.01$). Kaplan-Meier survival analysis using clinical samples revealed an association between shorter survival and high expression of LAMP-2A and LAMP-2B, but not LAMP-2C, in patients with RCC treated with sunitinib ($P=0.01$). Furthermore, high expression of LAMP-2A and LAMP-2B in RCC revealed a weak to moderate inverse correlation with the tumor shrinkage rate and progression-free survival, respectively. Thus, high expression of LAMP-2A and LAMP-2B contributed to the acquisition of sunitinib resistance, indicating that the expression of these two variants can predict the efficacy of sunitinib treatment in patients with RCC.

## Introduction

Renal cancer, the seventh most commonly encountered cancer, is responsible for more than 140,000 deaths annually worldwide (1). Approximately 20% of patients with renal cell carcinoma (RCC) are diagnosed at advanced stages (2). Localized RCC can be treated by surgical resection; however, metastatic RCC (mRCC) results in poor prognosis. Over the past decade, progress has been made in the treatment of advanced-stage RCC using molecular targeted therapy. Sunitinib malate, a tyrosine kinase inhibitor with antitumor and antiangiogenic activities, is a widely recognized (according to the 2018 National Comprehensive Cancer Network guidelines for kidney cancer management) first-line treatment option for patients with advanced-stage mRCC and unresectable RCC (3). The efficacy and safety of sunitinib have been evaluated in numerous clinical trials (4-6). Moreover, in the first-line setting, the efficacy of sunitinib was confirmed by clinical trials (4,7-11). However, most patients eventually develop resistance to sunitinib therapy, and the median progression-free survival (PFS) of patients following first-line treatment with sunitinib ranges between 9 and 11 months (4,7,9-11). Approximately 70% of patients initially respond to therapy, while the remaining 30% exhibit primary resistance to sunitinib. Among patients who respond to initial therapy, durable responses are rare, and most cases acquire sunitinib resistance within 6-15 months (12). The precise biological mechanism underlying sunitinib resistance remains unclear.

We have previously used ACHN cells, a human RCC cell line, to establish a sunitinib-resistant model (SR-ACHN) for examining the mechanism underlying sunitinib resistance (13,14). In SR-ACHN cells, the expression level of microRNA (miR)-194-5p was lower than that in ACHN cells, whereas the expression level of lysosome-associated membrane protein 2 (LAMP-2), one of the miR-194-5p target genes, was increased, suggesting that LAMP-2 plays a role in the acquisition of sunitinib resistance in RCC cells. LAMP-2, a major lysosomal membrane protein with a single transmembrane region, contributes to lysosomal function (15). There are three splice variants of LAMP-2, namely, LAMP-2A, LAMP-2B, and LAMP-2C, which have different physiological functions. However, the variant primarily responsible for sunitinib resistance in human RCC cells remains to be identified.

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*Key words:* drug resistance, renal cell carcinoma, lysosome-associated membrane proteins 2, sunitinib, splice site variant

The present study aimed to identify which LAMP-2 splice variants were involved in sunitinib resistance of RCC cells and potential biomarkers of sunitinib resistance in patients with mRCC.

## Materials and methods

*RCC cell lines*. ACHN, a parental human RCC cell line, was purchased from the American Type Culture Collection and cultured in the Roswell Park Memorial Institute (RPMI)-1640 (Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). Sunitinib-resistant ACHN (SR-ACHN) cells were established based on previously described methods (13). SR-ACHN cells were maintained in the same medium containing 10 $\mu$M sunitinib (Tocris Bioscience).

*Plasmid vectors and establishment of LAMP-2-overexpressing ACHN cells*. The following LAMP-2 expression vectors were used: LAMP-2A (cat. no. RC221216; OriGeneTechnologies, Inc.), LAMP-2B (cat. no. 86029) and LAMP-2C (cat. no. 89342; both from Addgene, Inc.). Plasmids with deletions of each LAMP-2 cDNA, pCMV-Entry, pCDNAHygro(+), and pCDNAZeo(-), were used as control vectors. For transfection, ACHN cells were seeded into 6-cm dishes at a density of $4x10^5$ cells per dish in a total volume of 3 ml. After the cells were cultured overnight, they were transfected with each plasmid vector using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

The LAMP-2A expression plasmid or its control plasmid-transfected ACHN was selected using 300 $\mu$g/ml G418 (Nacalai Tesque, Inc.). LAMP-2B expression plasmid- or its control plasmid-transfected ACHN was selected using 200 $\mu$g/ml hygromycin (Nacalai Tesque, Inc.), and the LAMP-2C expression plasmid or its control plasmid-transfected ACHN was selected using 80 $\mu$g/ml zeocin (Thermo Fisher Scientific, Inc.).

*RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA from the cell lines was extracted using the TRIzol® Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Next, 0.5 $\mu$g of RNA was reverse-transcribed into complementary DNA using PrimeScript™ RT Master Mix (Perfect Real Time; Takara Bio, Inc.) in a 10-$\mu$l reaction. The relative expression levels of LAMP-2A, LAMP-2B, and LAMP-2C mRNA were measured by quantitative PCR using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, Inc.) according to the manufacturer's instructions. Each 15-$\mu$l reaction contained 7.5 $\mu$l of the TB Green® Premix, 15 pmol primers (Table I), and 3.5 $\mu$l of the complementary DNA template, as well as a no-template control. Amplification was performed in 40 cycles of denaturation (95˚C, 15 sec), annealing, and extension (60˚C, 1 min). A complementary DNA template-free control was included for each PCR amplification. Gene expression was normalized to β-actin mRNA levels, and relative gene expression was calculated using the comparative quantification cycle (ΔΔCq) method (16).

*Cell proliferation assay*. Cells overexpressing LAMP-2A, LAMP-2B, and LAMP-2C were harvested and seeded into a 96-well plate ($5x10^3$ cells/well), and sunitinib was added at various concentrations (2.5, 5.0, 10, 20, 40 and 80 $\mu$M). The plates were incubated for 72 h, and the cells were fixed with 5% glutaraldehyde for 30 min at room temperature and then stained with 0.2% crystal violet with CAPS buffer (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. The crystal violet staining was dissolved with 10% acetic acid and quantified using a microplate reader at 495 nm. The results of the assay were the average value obtained from three experiments.

*Clinical samples*. Ten samples from patients with RCC who underwent nephrectomy between May 2010 and June 2017 and were treated with sunitinib following surgery due to metastases were obtained from the Tottori University Hospital. The median age of the 10 patients was 62 years (age range, 26-77 years); six were male and four were female (Table II). All materials were obtained with written informed consent, and the procedures were approved by the Ethics Committee of the Tottori University (Tottori, Japan; approval no. 1558). Tissue samples were obtained from tumor tissues of patients with RCC who underwent nephrectomy. The sunitinib response was evaluated using the Response Evaluation Criteria in Solid Tumors v1.1 (17). Most patients had received other molecular target drugs or immune checkpoint inhibitors before or after sunitinib treatment, but sunitinib was used as a single agent only and not as a combination therapy. Therefore, overall survival was not examined in the present study, but the effect of sunitinib on PFS and tumor reduction was examined. It has been suggested that immune checkpoint inhibitors may remain active even after administration has been completed. However, the two patients treated with nivolumab, an immune checkpoint inhibitor, both received it after sunitinib treatment; hence, treatment with nivolumab did not affect the efficacy of sunitinib.

*Statistical analysis*. The unpaired Student's t-test was used to detect significant changes in the expression levels of LAMP-2 splice variants, $IC_{50}$ for the cell proliferation assay, and gene expression in clinical samples. The Bonferroni correction was used to compare the $IC_{50}$ in multiple cell lines and relative expression levels of LAMP-2 splice variants. Pearson's correlation coefficient was used to evaluate the correlation between gene expression in clinical samples and their PFS or tumor shrinkage rate. The treatment effect of sunitinib was categorized into two groups based on the best overall response of each sample: i) the partial response (PR) group (n=3) and ii) the stable disease (SD) and progressive disease (PD) group (SD + PD; n=7). Kaplan-Meier survival analysis was utilized to evaluate the association of the expression levels of LAMP-2 splice variants with PFS using the log-rank test. To examine the relationship between survival curves and expression levels of LAMP-2 splice variants by categorizing 10 samples into two groups based on their expression levels, we defined the high expression group as samples with expression levels above the upper quartile and the low expression group as samples with expression levels below the upper quartile, following a previous study (18). All statistical analyses were performed with SPSS v23.0.0.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Quantitative PCR primer sequences.

| Target | Primer sequences (5'-3') | |
|---|---|---|
| LAMP-2A | F: GGGTTCAGCCTTTCAATGTG | R: CAGCATGATGGTGCTTGAGA |
| LAMP-2B | F: GGGTTCAGCCTTTCAATGTG | R: CCTGAAAGACCAGCACCAAC |
| LAMP-2C | F: GTATTCTACAGCTGAAGAATGTTCTG | R: ACACCCACTGCAACAGGAAT |
| β-actin | F: CGTGGGCCGCCCTAGGCACCA | R:TTGGCTTAGGGTTCAGGGGGG |

PCR, polymerase chain reaction; LAMP-2, lysosome-associated membrane protein 2; F, forward; R, reverse.

Table II. Patients and tumor characteristics as well as responses to sunitinib treatment.

| Characteristics | n |
|---|---|
| Age (years) | |
| Median | 62 |
| Range | 26-77 |
| Sex | |
| Male | 6 |
| Female | 4 |
| Histopathology | |
| Clear cell | 5 |
| Papillary | 3 |
| Chromophobe | 1 |
| Spindle | 1 |
| Pathological stage | |
| pT3 | 6 |
| pT2 | 0 |
| pT1 | 4 |
| Grade | |
| G3 | 5 |
| G2 | 5 |
| G1 | 0 |
| Metastatic sites | |
| Lung | 6 |
| Pleura | 1 |
| Liver | 3 |
| Pancreas | 1 |
| Adrenal gland | 1 |
| Brain | 1 |
| Lymph node | 3 |
| Bone | 3 |
| Best overall response | |
| PR | 3 |
| SD | 6 |
| PD | 1 |

PR, partial response; SD, stable disease; PD, progressive disease.

Table III. Comparison of resistance to sunitinib.

| Groups | Average $IC_{50}$ ± SD | P-value |
|---|---|---|
| ACHN and SR-ACHN | | |
| ACHN | 2.58±0.17 | - |
| SR-ACHN | 6.62±1.34 | <0.001 |
| LAMP-2A | | |
| 2A(-)1 | 2.50±0.08 | 0.422 |
| 2A(-)2 | 2.48±0.17 | 0.17 |
| 2A(+)1 | 3.52±0.17 | <0.001 |
| 2A(+)2 | 3.66±1.03 | 0.001 |
| LAMP-2B | | |
| 2B(-)1 | 2.91±0.86 | 0.172 |
| 2B(-)2 | 2.75±0.21 | 0.145 |
| 2B(+)1 | 3.87±0.05 | <0.001 |
| 2B(+)2 | 3.15±0.18 | <0.001 |
| LAMP-2C | | |
| 2C(-)1 | 2.63±0.19 | 0.618 |
| 2C(-)2 | 2.65±0.01 | 0.480 |
| 2C(+)1 | 2.63±0.19 | 0.643 |
| 2C(+)2 | 2.68±0.27 | 0.399 |

$IC_{50}$, half-maximal inhibitory concentration; SD, standard deviation; LAMP-2, lysosome-associated membrane protein 2.

## Results

*Establishment of ACHN cells overexpressing LAMP-2A, LAMP-2B, and LAMP-2C*. To investigate which LAMP-2 variants were required for acquisition of the sunitinib-resistant phenotype in human RCC cells, the LAMP-2A, LAMP-2B, or LAMP-2C expression vector or corresponding control vector was transfected into ACHN cells, and two representative clones were established in each transfection. RT-qPCR revealed that the expression levels of LAMP-2A, LAMP-2B, and LAMP-2C in the respective LAMP-overexpressing cells were higher than those in ACHN cells and control vector-transfected ACHN cells (Fig. 1). The LAMP-2 mRNA expression level in the control vector-transfected cells was similar to that in ACHN cells in each variant type.

*LAMP-2A and LAMP-2B expression contributes to sunitinib resistance in ACHN cells*. Table III revealed that the $IC_{50}$ values of sunitinib for ACHN and SR-ACHN cells were 2.58 and 6.62 $\mu$M, respectively, demonstrating that SR-ACHN cells exhibited significantly higher resistance to sunitinib

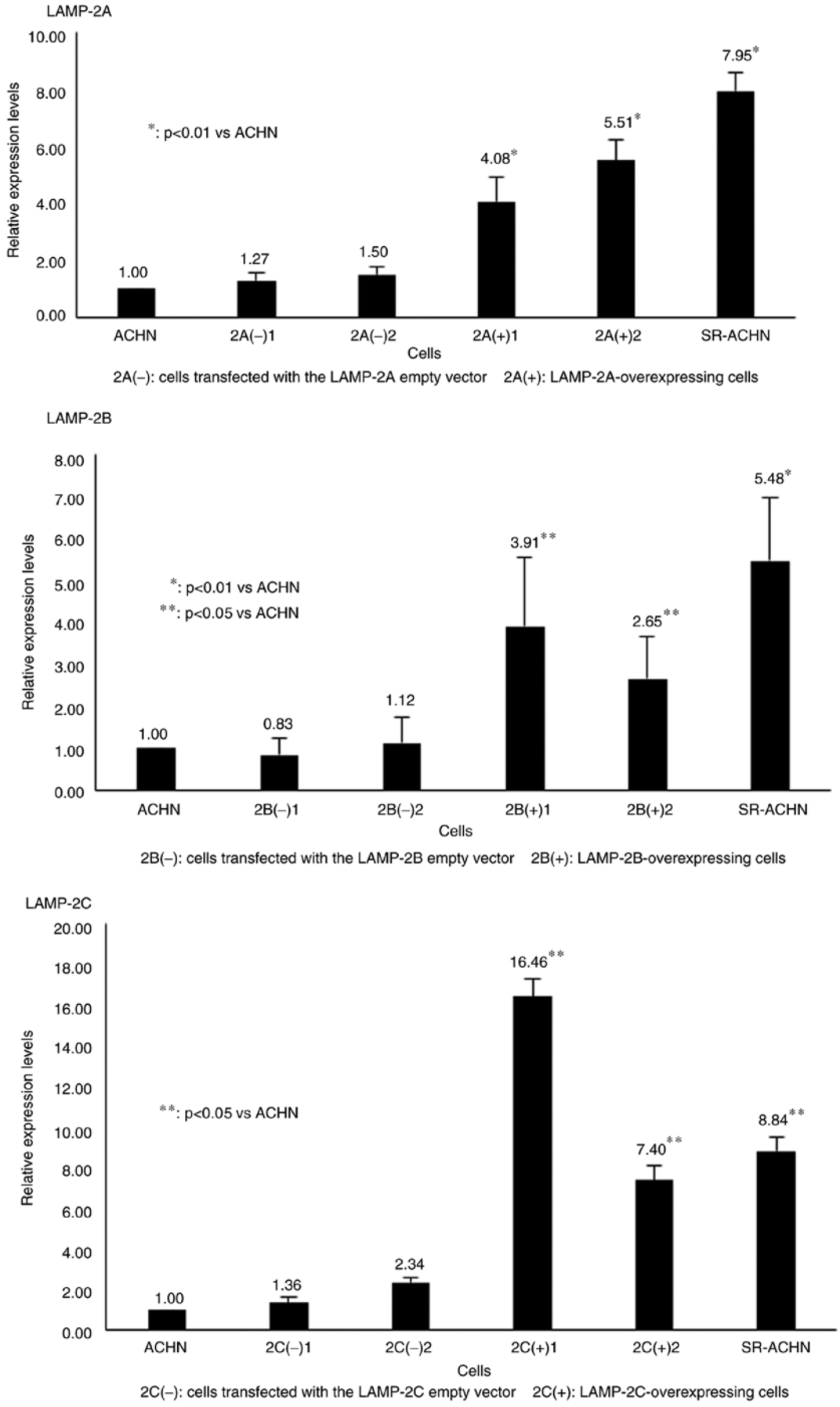


Figure 1. Established LAMP-2-overexpressing cells of each splice variant. The relative expression levels of each cell line compared to ACHN (=1.00) in qPCR are presented. LAMP-2A-, LAMP-2B-, and LAMP-2C-overexpressing cells had higher expression levels than did ACHN. Cell lines that had statistically significant increased expression compared to ACHN are indicated by *P<0.01 (as determined by the Bonferroni correction) and **P<0.05. LAMP-2, lysosome-associated membrane protein-2.

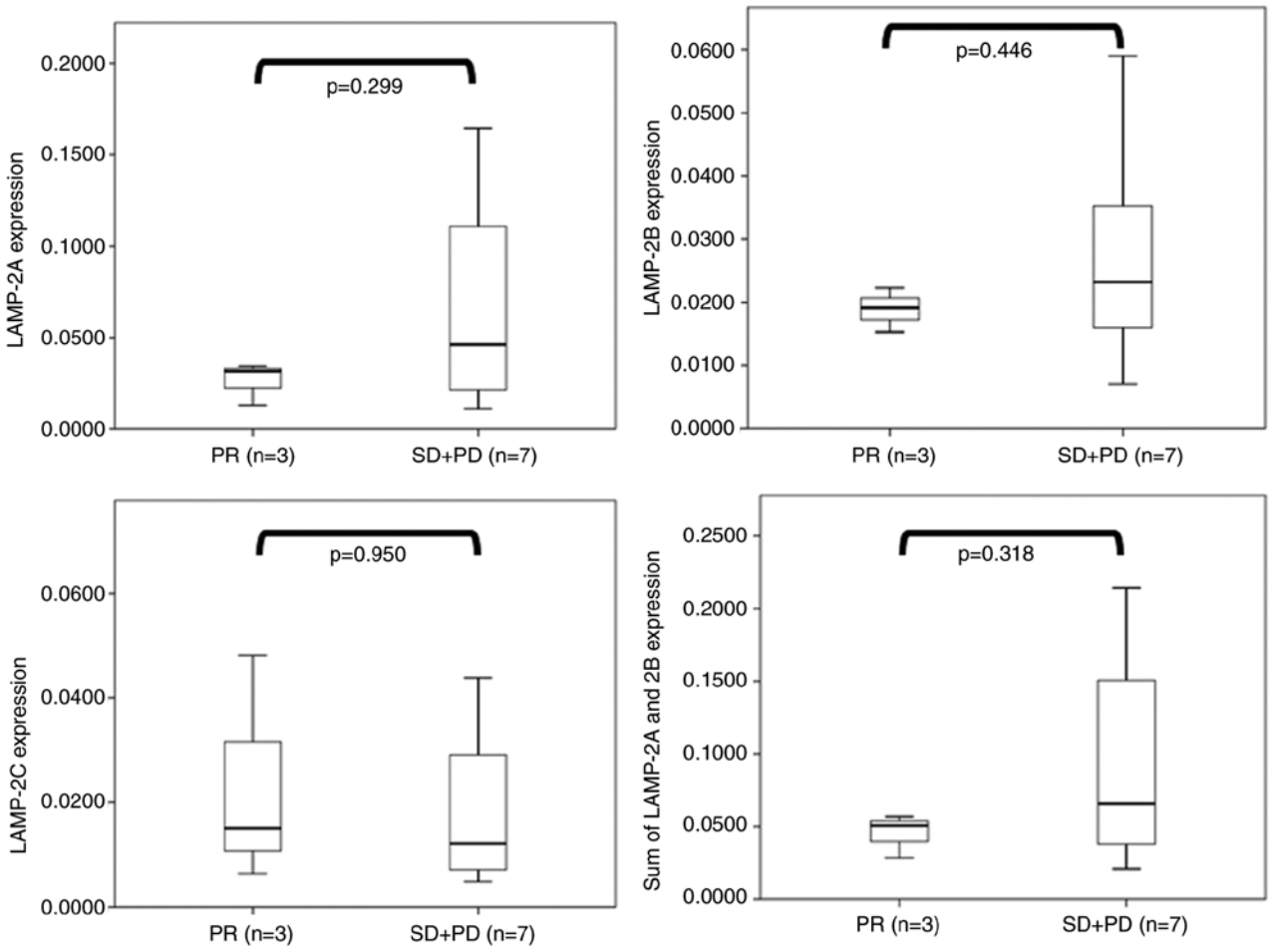


Figure 2. LAMP-2 splice variant expression in tumor tissue categorized into two groups based on achieving PR or SD/PD in the best overall response rate. Splice variants exhibited no significant differences. LAMP-2, lysosome-associated membrane protein 2; PR, partial response; SD, stable disease; PD, progressive disease.

treatment than did ACHN cells, as previously reported by Yamaguchi *et al* (13) and Yumioka *et al* (14). The $IC_{50}$ values in the two LAMP-2A-overexpressing ACHN clones [2A(+)1 and 2A(+)2] were 3.52 and 3.66 $\mu$M, respectively, significantly higher than those in ACHN cells (P<0.001). Moreover, the $IC_{50}$ values in the two LAMP-2B-overexpressing ACHN clones [2B(+)1 and 2B(+)2] were 3.87 and 3.15 $\mu$M, respectively, also significantly higher than those in ACHN cells (P<0.001). In contrast, the $IC_{50}$ values in the two LAMP-2C-overexpressing ACHN clones [2C(+)1 and 2C(+)2] were similar to those in ACHN cells (2.63 and 2.68 $\mu$M, respectively). The $IC_{50}$ values in all control vector-transfected clones were nearly equal to those in ACHN cells.

*Expression of LAMP-2A and LAMP-2B in clinical samples.* To evaluate the correlation between the expression levels of LAMP-2A, LAMP-2B, and LAMP-2C and susceptibility to sunitinib in clinical samples, qPCR was performed using RNA derived from 10 RCC clinical samples. The backgrounds, clinicopathological features, characteristics of the metastatic sites, and therapeutic effect of patients are presented in Table II.

As revealed in Fig. 2, there was no significant difference between the therapeutic effect and expression levels of any LAMP-2 variants. However, LAMP-2A, LAMP-2B, and the sum of LAMP-2A and LAMP-2B cases exhibiting high expression were revealed in only the SD + PD groups, but not in the PR groups. In contrast, no difference in LAMP-2C expression was observed between the SD + PD and PR groups.

Next, the correlation between the sunitinib treatment period PFS and the gene expression status was evaluated. LAMP-2A (r=-0.279; P=0.434) and LAMP-2B (r=-0.219; P=0.543) exhibited weak negative correlations between PFS and each gene expression level (Fig. 3A). In contrast, LAMP-2C exhibited no such correlation (r=-0.054; P=0.882). Additionally, there were moderate correlations between a high tumor shrinkage rate and a low expression of LAMP-2A (r=-0.442; P=0.201), as well as the concomitant sum of LAMP-2A and LAMP-2B expression (r=-0.430; P=0.215; Fig. 3B). There was a weak correlation between a high tumor shrinkage rate and a low expression of LAMP-2B (r=-0.346; P=0.328). Correlations among LAMP-2A, LAMP-2B, and LAMP-2C were also evaluated, revealing that their expression levels were in fact correlated, of which LAMP-2A and LAMP-2B exhibited the strongest correlation (Fig. 3C). Thus, although there were differences in the expression levels of the three splice variants of LAMP-2 in the 10 patients, all three variants were expressed at the mRNA level in all cases.

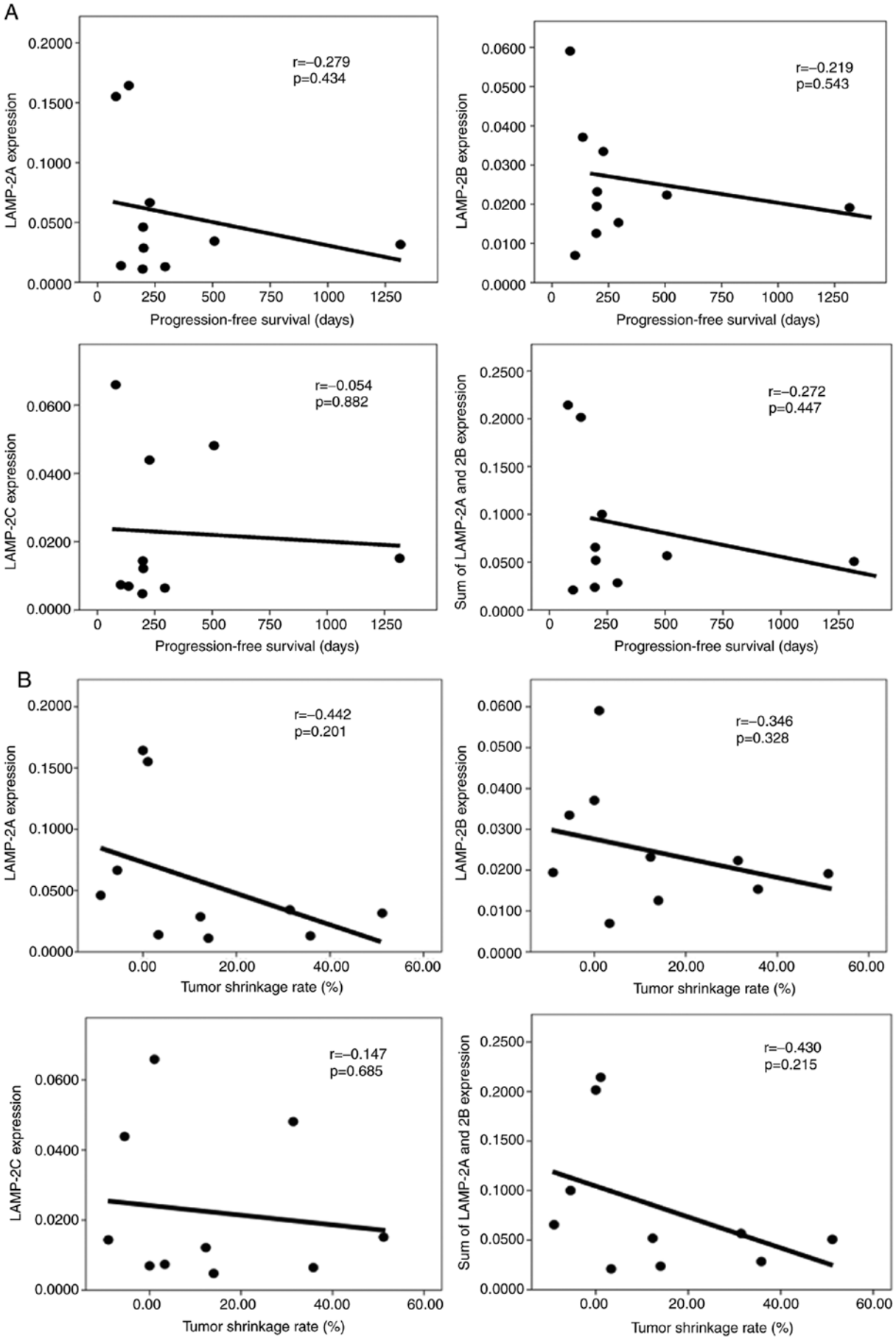


Figure 3. Correlation between the expression levels of LAMP-2 splice variant and (A) progression-free survival and (B) tumor shrinkage rate during sunitinib treatment. (A) LAMP-2A and LAMP-2B exhibited weak negative correlations between PFS and each gene expression level. (B) A moderate correlation was observed between a high tumor shrinkage rate and low expression of LAMP-2A and LAMP-2A plus LAMP-2B.

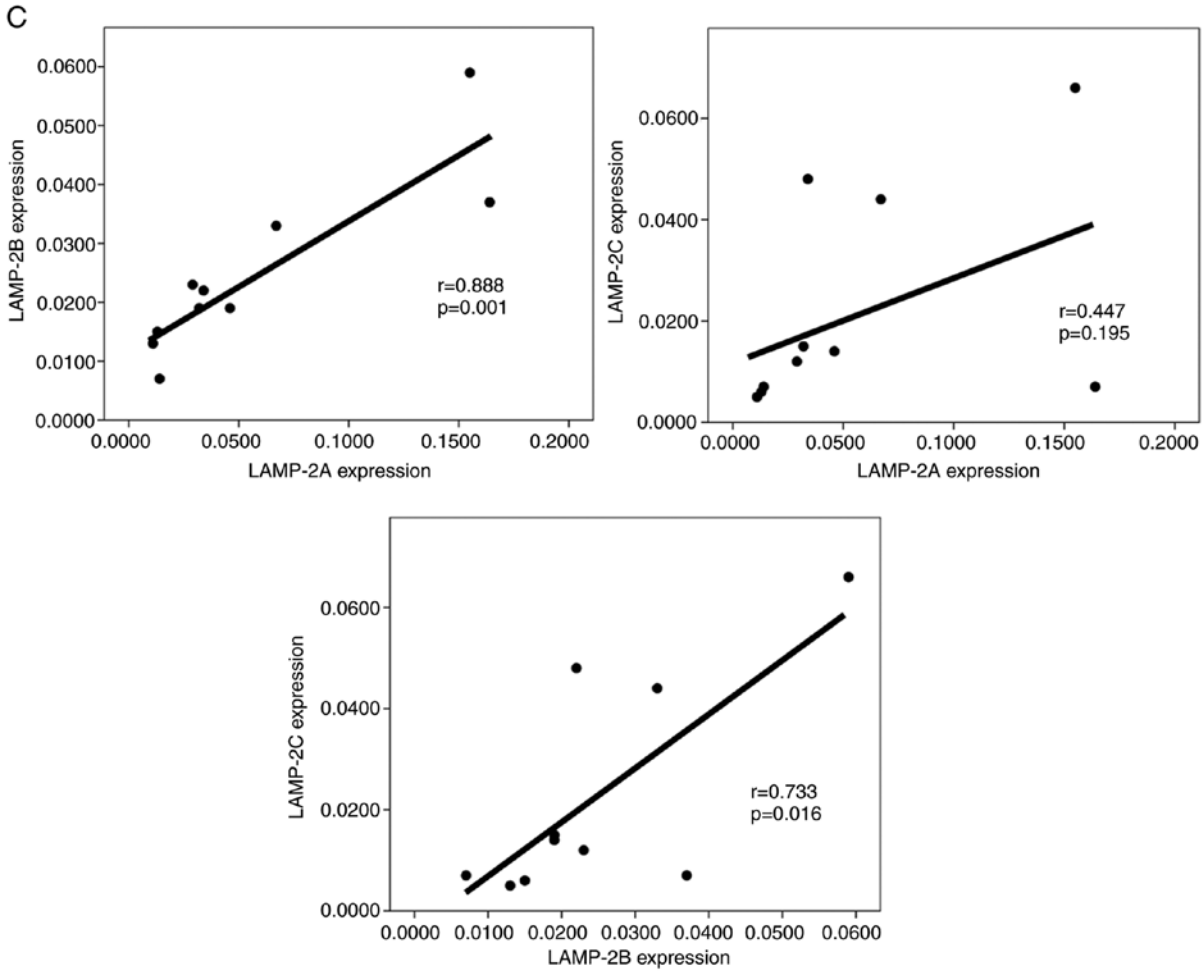


Figure 3. Continued. (C) Correlation of the expression levels of each splice variant of LAMP-2 in clinical samples. The strongest correlation was observed between 2A and 2B. LAMP-2, lysosome-associated membrane protein 2.

Kaplan-Meier plots were also utilized to evaluate the effect of LAMP-2 variant expression levels on sunitinib resistance (Fig. 4). In all LAMP-2 splice variants, there were 3 patients in the high expression group and 7 in the low expression group. For the LAMP-2A, LAMP-2B, and sum of LAMP-2A and LAMP-2B expression levels, the cases in the two groups of high and low expression levels were consistent, thus similar survival curves were obtained. Although LAMP-2C exhibited no difference between the high expression and the low expression groups (P=0.981), LAMP-2A, LAMP-2B, and the sum of LAMP-2A and LAMP-2B were significantly different between the two groups (P=0.010). The low expression group of these variants exhibited a significantly longer PFS.

The association between LAMP-2 splice variant expression levels and tumor pathological grades was then examined (Fig. 5A) and no significant difference between pathological grades 2 and 3 were revealed. However, tumors with a higher pathological grade tended to exhibit higher expression levels of LAMP-2A (P=0.131) and LAMP-2B (P=0.093). The associations between pathological T stage and LAMP-2 splice variants in resected tumors were also examined (Fig. 5B). However, there was no significant difference between the pathological stage (pT1, n=3 vs. pT3, n=7) and the expression levels of all splice variants of LAMP-2.

## Discussion

In the present study, it was demonstrated that LAMP-2A and LAMP-2B were involved in sunitinib resistance in RCC cells (12). Despite its therapeutic effect in patients with advanced-stage RCC, sunitinib use remains limited since tumors acquire drug resistance early on. Gotink *et al* reported that increased sequestration of sunitinib in lysosomes enhanced by the overexpression of LAMP-2 resulted in sunitinib resistance in RCC cells (19). We also previously revealed that LAMP-2 is involved in sunitinib resistance in RCC cells (13,14); however, we did not examine which splice variants (2A, 2B, and 2C) contributed to such resistance. LAMP-2A and LAMP-2B are constitutively expressed in most tissues and cells, whereas high expression of LAMP-2C is limited to the brain, heart, skeletal muscle, small intestine, and colon (20). However, their functions in the acquisition of drug resistance in cancer cells remain unclear.

To the best of our knowledge, this is the first study regarding the influence of LAMP-2A and LAMP-2B on sunitinib resistance in RCC cells. LAMP-2A was reported to be a key protein in chaperone-mediated autophagy (21). In contrast, LAMP-2B was revealed to be involved in macroautophagy, and Danon disease is caused by loss of the LAMP-2B isoform (22). As both variants are involved in autophagy, the present results

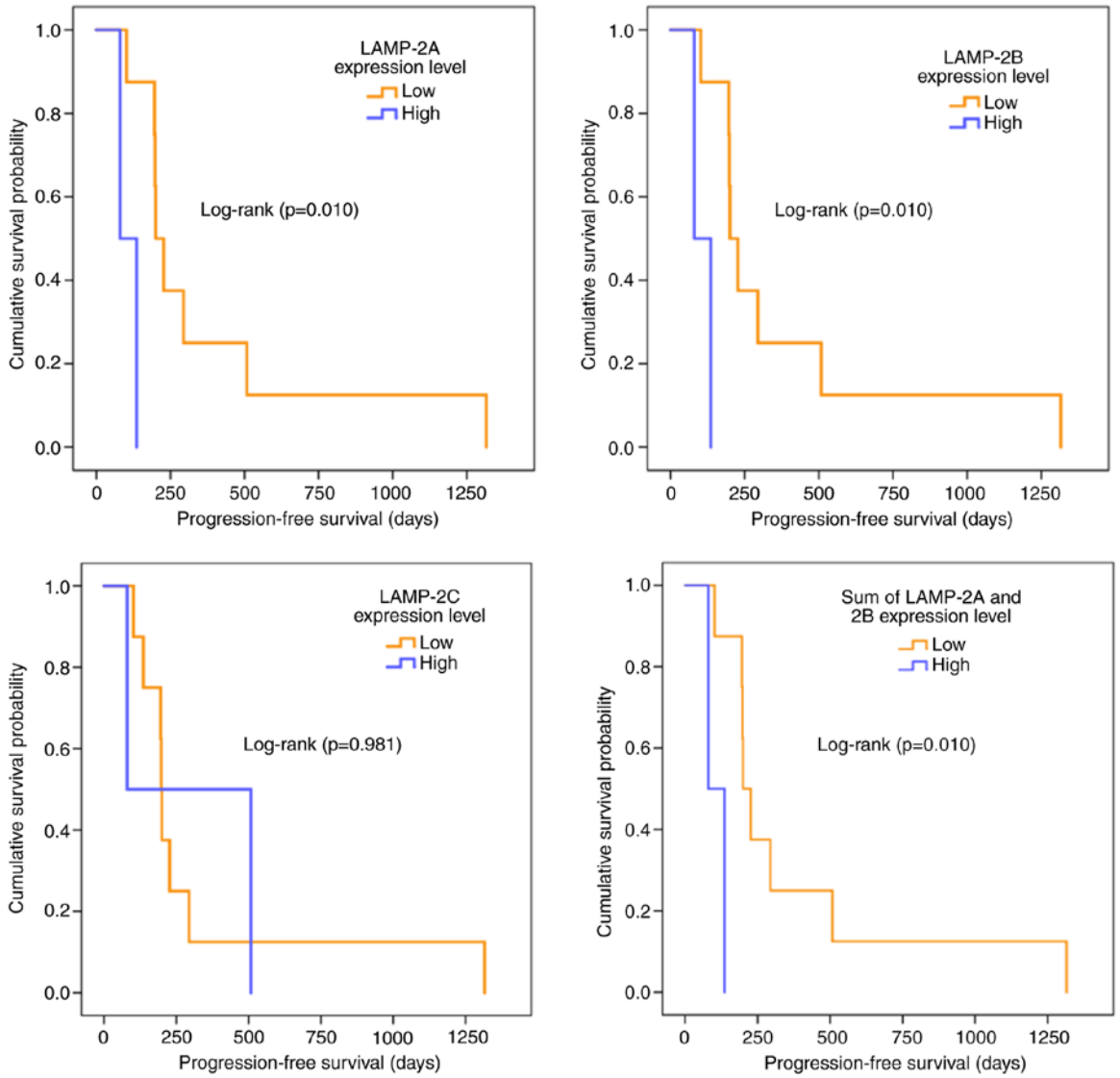


Figure 4. Kaplan-Meier plots and survival analysis of LAMP-2 splice variant expression levels categorized into two groups: High or low expression. A high expression level was defined as being above the upper quartile (n=3) and a low expression level was defined as being below the upper quartile (n=7). For the LAMP-2A, LAMP-2B, and sum of LAMP-2A and LAMP-2B expression levels, the cases in the two groups of high and low expression levels were consistent, thus similar survival curves were obtained. Statistically significant differences were detected between the two groups for these splice variants of LAMP-2 except for LAMP-2C. LAMP-2, lysosome-associated membrane protein 2.

indicated that the acquisition of sunitinib resistance in RCC cells is possibly mediated by autophagy activation. Autophagy is reported to affect both cancer cell growth and death, depending on the tissue environment (23). It has been suggested that chemotherapy-induced autophagy results in the acquisition of resistance against therapy-mediated cell death (24,25). In anti-angiogenesis therapy, such as sunitinib treatment, the upregulation of autophagy caused sunitinib resistance in patients with RCC (26). Giuliano *et al* reported that sunitinib resistance of RCC cells was caused by inappropriate autophagy flux via sequestration of sunitinib in lysosomes, suggesting that LAMP-2 was involved in the mechanism (27).

Strategies for overcoming sunitinib resistance have been developed, such as combination therapy, sunitinib re-challenge, sequential therapy, and dose-escalation in both animal and human studies (12,28). Sasaki *et al* reported that chloroquine (CQ), an anti-malarial agent and autophagy inhibitor, potentiates the anticancer effect of 5-fluorouracil on colon cancer cells (29), and Li *et al* reported that CQ, in combination with sunitinib, could enhance the anti-RCC effects of sunitinib (30). In fact, the therapeutic effects of sunitinib were enhanced when combined with CQ against pancreatic neuroendocrine tumors via autophagy inhibition and lysosomal membrane permeabilization (31). In this study, it was also indicated that combination with CQ was as effective as LAMP-2 knockdown. These studies strongly indicate that autophagy is involved in sunitinib resistance, suggesting that the overexpression of LAMP-2A and LAMP-2B plays a role in the acquisition of sunitinib resistance in RCC cells. Conversely, LAMP-2C does not appear to be involved in sunitinib resistance in RCC cells. Fujiwara *et al* reported that LAMP-2C serves as a receptor for RNautophagy and DNautophagy, in which RNA and DNA are taken up directly into lysosomes for degradation (32,33). LAMP-2C may not be involved in taking up drugs, including sunitinib, into lysosomes, and thus, it is considered that LAMP-2C is not involved in the acquisition of sunitinib resistance of RCC cells.

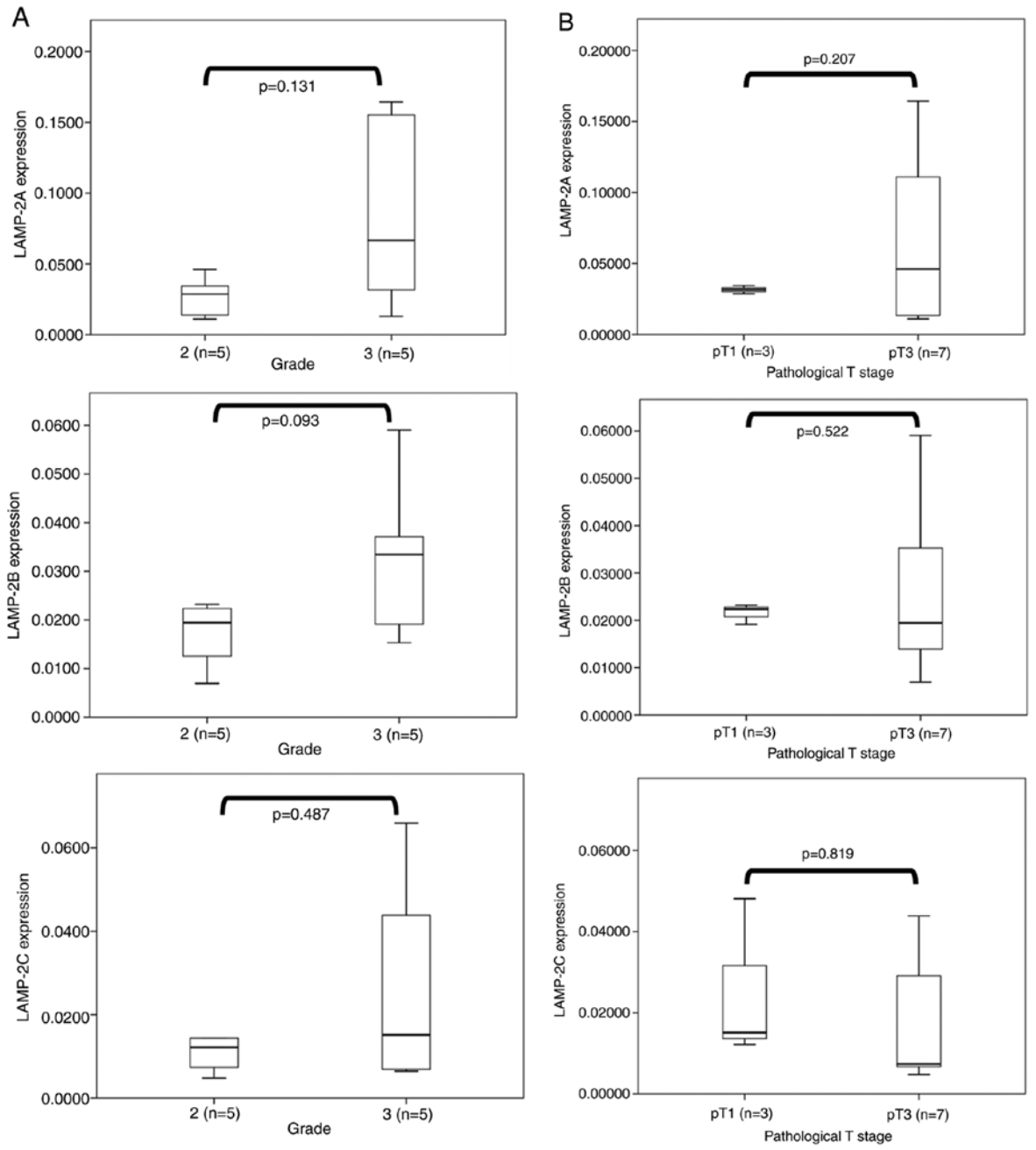


Figure 5. Pathological grades and LAMP-2 variant expression levels (A) Pathological higher-grade tumors tended to exhibit higher expression levels of LAMP-2A and LAMP-2B. Pathological stages and LAMP-2 variant expression levels (B) LAMP-2, lysosome-associated membrane protein 2.

In clinical samples of patients with RCC who were treated with sunitinib for metastases following nephrectomy at our hospital, Tottori University Hospital, higher expression of LAMP-2A and LAMP-2B was indicated to be involved in the response to sunitinib. In terms of the best overall response rate, the expression levels of LAMP-2A and LAMP-2B tended to be associated with the overall response rate, although there was no significant difference in these groups. In addition, high gene expression levels and shorter PFS during the sunitinib treatment period were weakly associated with both LAMP-2A and LAMP-2B. The tumor-reducing effect of sunitinib treatment was also moderately associated with LAMP-2A and weakly associated with LAMP-2B. Moreover, patients exhibiting high expression levels of these two splice variants also exhibited significantly lower survival rates in the Kaplan-Meier curve analysis. These clinical sample data are strongly consistent with the *in vitro* data, suggesting that high expression of LAMP-2A and LAMP-2B contributes to the prognosis of sunitinib-treated patients with RCC via acquisition of sunitinib resistance.

There are several limitations to the present study. One is that only one cell line was used in this experiment. The involvement of the LAMP-2 splice variants in sunitinib resistance may have been detected only in ACHN cells and not in other RCC cell lines. In addition, future investigations by animal studies are required. Future studies should involve examination of subcutaneous transplantation of LAMP-2A- and LAMP-2B-overexpressing RCC cell lines into mice, with differences in growth rates between normal cells and LAMP-2-overexpressing cells

after sunitinib treatment. The involvement of LAMP-2A and LAMP-2B in sunitinib resistance needs to be confirmed using *in vitro* studies as well. Another limitation of this study is the small number of clinical samples examined for clinicopathological analysis. It has been difficult to obtain a larger clinical sample at our institution due to the small number of patients with a history of nephrectomy that have used sunitinib; thus, future studies should involve larger sample sizes.

LAMP-2A and LAMP-2B were revealed to be involved in the acquisition of sunitinib resistance in patients with RCC possibly through the mediation of autophagy, strongly suggesting that their expression in RCC can be used as a predictive marker for sunitinib resistance. Moreover, since their low expression levels may be associated with enhanced antitumor effects of sunitinib and contribute to tumor reduction and longer PFS, the development of LAMP-2A and LAMP-2B inhibitors may help overcome the acquisition of sunitinib resistance in RCC.

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author upon a reasonable request. Patient data are confidential and therefore have not been shared.

**Authors' contributions**

RN contributed majorly towards the writing of the manuscript. MO supervised the experiments and assisted with the writing of the manuscript. RN, RS, and MI performed the experiments and collected the data. TK provided the reagents needed for the experiments. RN, TY, NY and HI obtained the tumors and tissues with their clinical information when available. RN and RS performed the statistical analysis. RN, MO and MH analyzed the data and drafted the manuscript. AT and FO made contributions to the design of the study and critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All RCC tissues were obtained with informed consent, and the procedures were approved by the Ethics Committee of Tottori University (Tottori, Japan; approval no. 1558).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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