Serine Threonine Kinase 11/Liver Kinase B1 Mutation in Sporadic Scirrhous-type Gastric Cancer Cells

Sadaaki Nishimura, Masakazu Yashiro, Tomohiro Sera, Yurie Yamamoto, Yukako Kushitani, Atsushi Sugimoto, Shuhei Kushiyama, Shingo Togano, Kenji Kuroda, Tomohisa Okuno, Yoshiki Murakami, Masaichi Ohira

Highlights	【研究のポイント】 ◇ スキルス胃癌について、今まで解明されていなかった原因遺伝子と治療標的分子を同定した世界初の研究。 ◇ スキルス胃癌の治療が大きく前進する可能性を示唆。
Description	【概要】 研究グループは、スキルス胃癌の原因変異遺伝子として STK11/LKB1 を同定し、スキルス胃癌治療薬として STK11/LKB1 関連シグナル系を制御するラパマイシンが有用であることを明らかにしました。 スキルス胃癌は、急速に増殖進展し、高頻度に転移する極めて難治性の癌とされています。しかし、これまでその原因遺伝子や治療標的分子は十分に解明されていませんでした。 今回、同研究チームは、附属病院で樹立したスキルス胃癌細胞株 6 株を用いて遺伝子解析を試みたところ、6 株中 3 株に癌抑制遺伝子 STK11/LKB1 の変異を同定しました。さらにサンガーシークエンス法で同遺伝子の変異を確認すると、STK11/LKB1 変異型スキルス胃癌細胞では STK11/LKB1 蛋白質の発現減弱や機能喪失も確認されました。これを受けて、胃癌細胞株に STK11/LKB1 関連シグナル系を制御するラパマイシンを作用させたところ、スキルス胃癌細胞の増殖が抑制されました。 スキルス胃癌における原因遺伝子と治療標的分子を解明した世界初の研究であり、この研究成果によって、スキルス胃癌の治療が大きく前進すると考えられます。 ‘スキルス胃癌の原因変異遺伝子の同定に成功！’. 大阪市立大学. https://www.osaka-cu.ac.jp/ja/news/2020/200403. (参照 2020/04/03)

Sadaaki Nishimura, Masakazu Yashiro, Tomohiro Sera, Yurie Yamamoto, Yukako Kushitani, Atsushi Sugimoto, Shuhei Kushiyama, Shingo Togano, Kenji Kuroda, Tomohisa Okuno, Yoshiki Murakami, Masaichi Ohira. (2020). *Serine Threonine Kinase 11/Liver Kinase B1* Mutation in Sporadic Scirrhous-type Gastric Cancer Cells. *Carcinogenesis*, 41. 1616-1623. Doi: 10.1093/carcin/bgaa031

Serine Threonine Kinase 11/Liver Kinase B1 Mutation in Sporadic Scirrhous-type Gastric Cancer Cells

Sadaaki Nishimura[1,2,3], Masakazu Yashiro[1,2,3 *], Tomohiro Sera[1,2,3], Yurie Yamamoto[2,3], Yukako Kushitani[1,2,3], Atsushi Sugimoto[1,2,3], Shuhei Kushiyama[1,2,3], Shingo Togano[1,2,3], Kenji Kuroda[1,2,3], Tomohisa Okuno[1,2,3], Yoshiki Murakami[4], Masaichi Ohira[2]

1. Molecular Oncology and Therapeutics, Osaka City University Graduate School of Medicine, Osaka, Japan.
2. Department of Gastroenterological Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan.
3. Cancer Center for Translational Research, Osaka City University Graduate School of Medicine, Osaka, Japan.
4. Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan.

Corresponding to:

Masakazu Yashiro M.D.

Molecular Oncology and Therapeutics, Osaka City University Graduate School of Medicine

1-4-3, Asahi-machi, Abeno-ku, Osaka City, Osaka 545-8585, Japan

TEL; (+81) 6-6645-3838

FAX; (+81) 6-6646-6450

e-mail address; m9312510@med.osaka-cu.ac.jp

ABSTRACT

Scirrhous-type gastric carcinoma (SGC), which is characterized by the rapid proliferation of cancer cells accompanied by extensive fibrosis, shows extremely poor survival. A reason for the poor prognosis of SGC is that the driver gene responsible for SGC has not been identified. To identify the characteristic driver gene of SGC, we examined the genomic landscape of six human SGC cell lines of OCUM-1, OCUM-2M, OCUM-8, OCUM-9, OCUM-12, and OCUM-14, using multiplex gene panel testing by next-generation sequencing. In this study, the nonsynonymous mutations of *serine threonine kinase 11/liver kinase B1* (*STK11/LKB1)* gene were detected in OCUM-12, OCUM-2M, and OCUM-14 among the six SGC cell lines. Capillary sequencing analysis confirmed the nonsense or missense mutation of *STK11/LKB1* in the 3 cell lines. Western blot analysis showed that LKB1 expression was decreased in OCUM-12 cells and OCUM-14 cells harboring *STK11/LKB1* mutation. The mammalian target of rapamycin (mTOR) inhibitor significantly inhibited the proliferation of OCUM-12 and OCUM-14 cells. The correlations between STK11/LKB1 expression and clinicopathologic features of gastric cancer were examined using 708 primary gastric carcinomas by immunochemical study. The low STK11/LKB1 expression group was significantly associated with SGC, high invasion depth, and frequent nodal involvement, in compared to the high STK11/LKB1 expression group. Collectively, our study demonstrated that *STK11/LKB1* mutation might be responsible for the progression of SGC, and suggested that mTOR signaling by *STK11/LKB1* mutation might be one of therapeutic targets for patients with SGC.

Summary

- *Serine Threonine Kinase 11/Liver Kinase B1* (*STK11/LKB1*) was mutated in 3 of 6 SGC cell lines.
- Low STK11/LKB1 expression level was recognized in most of patients with SGC.
- The mTOR signaling by *STK11/LKB1* mutation might be one of therapeutic targets for this intractable disease of SGC**.**

INTRODUCTION

Scirrhous-type gastric carcinoma (SGC), diffusely infiltrating gastric carcinoma, or Borrmann type 4 (also known as linitis plastica-type carcinoma) is characterized by rapid cancer cell infiltration and proliferation accompanied by extensive stromal fibrosis (1-3). SGC accounts for approx. 10% of all gastric cancers (GCs), and the survival of patients with SGC remains extremely poor (1,3). It has been reported that the neoadjuvant chemotherapy for patients with SGC (JCOG0501) could not find a survival benefit (4). One of the reasons for the poor prognosis of SGC is the lack of effective therapeutic agents based on the characteristic molecular biology of SGC (5,6). Then, in this study, we aimed to find the characteristic features and useful therapeutic targets for SGC.

Several studies have defined the genomic landscape of GC using the next-generation sequencing (NGS) technology and have detected some candidates for driver gene of GC (7-11). It has been well-known that the genetic alternations of *TP53* and *CDH1* were associated with GC, including SGC (11). Currently, it has been reported that *RHOA* and *KMT2C* were characteristic mutated genes in SGC (12,13), however, no targeting drug against *RHOA* and *KMT2C* mutations is not available, so far. Novel molecular target therapy is urgently necessary for SGC based on its biological behavior (1,14,15), but the frequency of actionable gene mutations of SGC is low (10,16).-In our prior research, we established six SGC cell lines derived from SGC and demonstrated the characteristic features of these cells (2,14,16). In this context, using these SGC cell lines, we investigated the genetic alterations of SGC cells by using an NGS multiplex gene panel in this study. We observed that *serine threonine kinase11* (*STK11*), also known as *liver kinase B1* (*LKB1*), was frequently mutated and inactivated in SGC. A germline mutation of *STK11/LKB1* was identified as the genomic alternation responsible for Peutz-Jeghers syndrome, in which carriers show polyposis of the gastrointestinal tract as well as cancer of different organs (17,18). However, somatic

mutations of *STK11/LKB1* are rare in gastrointestinal cancer (8,9,19). We then studied the role of *STK11/LKB1* mutation in SGC cell lines, and examined the clinicopathological significance of STK11/LKB1 expression in gastric carcinomas in this study. Since STK11/LKB1 inactivation has reported to stimulate the mammalian target of rapamycin (mTOR) signaling (20,21), we examined the effect of mTOR inhibitor on the proliferation of SGC cells with *STK11/LKB1* mutation.

MATERIALS and METHODS

Sample and DNA extraction

A total of 6 SGC cell lines(2), OCUM-1 (22), OCUM-2M (23), OCUM-8(24), OCUM-9(24), OCUM-12(25), OCUM-14(14), which were established at our laboratory, were incubated in culture medium consisted of Dulbecco's modified Eagle's medium (DMEM; Nikken, Kyoto, Japan) with the addition of 10% fetal bovine serum (Nichirei, Tokyo, Japan), 100 IU/mL penicillin (Wako, Osaka, Japan), 100 mg/mL streptomycin (Wako), and 0.5 mmol/L sodium pyruvate (Wako). All cell lines in this study were authenticated by STR profiling before distribution. We tested for mycoplasma contamination in all cell lines and proved that there was no mycoplasma in cell lines. Cells were cultured at 37 C in 21% O_2. Each genomic DNA (gDNA) from 6 SGC cell lines was isolated using phenol-chloroform extraction. Purification and quantification of gDNA were conducted with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Qubit 4 Fluorometer (Thermo Fisher Scientific).

Library preparation and Next generation sequencing

A total of 50 ng of each gDNA was fragmented and then evaluated by the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA). Each sheared gDNA was used to perform end repair, A-tailing and adapter ligation with either Agilent SureSelect QXT (Agilent Technologies) to prepare sequencing libraries, following the manufacturer instructions. Subsequently, the libraries were captured using Agilent SureSelect NCC oncopanel (Agilent Technologies), targeted the coding exons of 90 cancer associated genes including 12 fusion genes with capturing 930 kp targeted exon region, and finally amplified.

After the enriched and amplified samples concentrations were determined using HSD1000 Screen Tapes (Agilent Technologies), target resequencing was performed using the Illumina Miseq platform (Illumina, San Diego, CA) with 150bp paired-end reads.

Bioinformatics analysis

SureCall software (version 4.0.1.46, Agilent Technologies) was used to trim and align fastq reads to the hg 19 reference genome with default parameters, detecting the variants, including single nucleotide variant (SNV) and InDel, of 90 genes in the panel, along with rearrangements of 12 oncogenes. The variants resulting from SureCall software was annotated and filtered using VariantStudio 3.0 (Illumina). Variants listed in >5% of 1000 genome database, the integrative Japanese Genome Variation Database and the Exome Aggregation Consortium Browser were excluded from further analysis, whereas genes mutation harboring a variant allele frequency down to 1% were included. Finally, filtered variants were annotated using the COSMIC database (Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cosmic). Copy number analysis was performed using CNVkit (26), which infer and visualize copy number variations from sequencing data with read-depth information. Normal reference used for copy number variant (CNV) identification were constructed using sequencing data from 15 frozen normal tissue of stomach in patients with gastric cancer. We also tried to detect fusion gene from panel-based NGS data using SureCall software.

Capillary sequencing analysis

Genomic DNA of the cell lines was extracted with phenol-chloroform. For *STK11/LKB1* gene was amplified by PCR for 35 cycles with Taq DNA polymerase on a thermal cycler. The following 2 primers were used for genomic *STK11/LKB1* PCR amplifications to detect the *STK11/LKB1* mutations: 5'-TCCTACCCCGTAGCCTCCACTA-3' and 5'-CAGAAGCAGGGTGGAGCTGAG-3': 5'-TCCTACCCCGTAGCCTCCACTA-3' and 5'-CAGAAGCAGGGTGGAGCTGAG-3'. PCR conditions were as follows: predenaturation, 94 °C for 10 min; denaturation, 94 °C for 1min; extension, 72 °C for 1 min; and final incubation, 72 °C for 10 min. The DNA templates were sequenced using BIG Dye terminators (version 3.1). PCR products were analyzed using a genetic analyzer (ABI PRISM 3130xl; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Western blot

OCUM-2M, OCUM-9, OCUM-12, and OCUM-14 cells were rinsed with PBS, and incubated in DMEM. After incubation, cell extracts (20 mg protein) were used for western blot analysis. The following primary antibodies were used: β-actin (1:3000; Sigma-Aldrich, St Louis, MO), and LKB1 (1:300; Santa Cruz, Dallas, TX).

Proliferation assay

The effect of mTOR inhibitor, Rapamycin, on the proliferation of SGC cells was determined by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) assay. A total of 1×10^3 SGC cells were seeded into two 96-well plates with culture medium exposed to Rapamycin at various concentrations (1×10^{-3} to 100 nmol/L).

Immunohistochemical analysis

The STK11/LKB1 expression was immunochemically examined using 708 gastric cancers taken by gastrectomy. Immunohistochemical staining was performed using LKB1 antibody (Santa Cruz, Dallas, TX) and phosphorylated-LKB1 (pLKB1) antibody (Santa Cruz). The immunohistochemical determination of LKB1 and pLKB1 was performed as described below. In brief, we performed deparaffinization and slides were heated for 10 min at 105°C in an autoclave in Target Retrieval Solution (Dako). After blocking endogenous peroxidase activity, the specimens were incubated with LKB1 antibody (1:150) and pLKB1 antibody (1:150) for 1 hour at room temperature. The slides were incubated with biotinylated anti-mouse IgG for 10 min. The slides were treated with streptavidin-peroxidase reagent, followed by counterstaining with Mayer's hematoxylin. All fields were analyzed at a magnification of × 200. The STK11/LKB1 expression was analyzed by intensity of staining and percentage of stained cancer cells as follows. 0 = no staining or weak staining in less than 10% of cancer cells; 1+ = weak staining in more than 10% of cancer cells or strong staining in less than 10% of cancer cells; 2+ = strong immunostaining in more than 10% of cancer cells. Scores of 0 or 1+ were identified as the STK11/LKB1-low expression group, whereas scores of 2+ were identified as the STK11/LKB1-high expression group. Evaluation was made by two double-blinded independent observers who were unaware of clinical data and outcome. When a different evaluation between the two independent observers was found, the evaluation was rechecked and argued. Then we analyzed the correlations between STK11/LKB1 expression and clinicopathologic features of gastric cancer.

Statistical analysis

Correlations between the high and low STK11/LKB1 expression group were determined using the chi-square test. Survival rates were estimated using the Kaplan-Meier method, and the differences in survival according to the group classification of patients were analyzed by log-rank test. Univariate and multivariate analyses were performed to determine significant prognostic factors using Cox regression models. The presence of a statistically significant difference was denoted by $p < 0.05$. All analyses were performed using GraphPad Prism 8.3.0 (GraphPad Software, La Jolla CA) or EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)(27). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

Ethics statement

This study was approved by Osaka City University Hospital Certified Review Board (Permission number: CRB5180003) and carried out according to the guidelines of the committee. Written informed consent was obtained from all individual participants included in the study. And this study has been conducted according to the principles of the declaration of Helsinki.

RESULTS

Single-nucleotide variants and indels in the SGC cell lines

We performed the target resequencing of six SGC cell lines by using the NCC Oncopanel™, which covers 90 cancer-associated genes. The target resequencing covered 98.92%–99.78% of the coding regions of the genes, and the sequencing yielded an average read depth of 63–362 reads within the region of interest (**Supplement table S1**). **Supplement table S2** lists all of the variants that were filtered by our bioinformatics analysis. A total of 33 genomic nonsynonymous alterations, excluding synonymous and in-frame variants, were detected in the six SGC cell lines (**Supplement Table S3**). Among these alterations, 21 mutations were listed on the COSMIC database; the remaining 12 mutations were not listed on a large database including the COSMIC and dbSNP databases (http://www.ncbi.nlm.nih.gov/snp/).

Figure 1A summarizes the mutated genes based on the alteration types in the six SGC cell lines. The most common nonsynonymous mutation was *TP53*, which was detected in four of the six SGC cell lines, and 50% of the *TP53* mutations were pathogenic mutations according to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), followed by *STK11/LKB1*, *ARID2*, and *ERBB2* in three of the six SGC cell lines. The nonsynonymous mutations of *CTNBB1*, *ARID1A*, and *PTCH1* were detected in two of the six SGC cell lines. The other nonsynonymous mutated genes were detected exclusively in one of the six cell lines. Rearrangement was not observed in any of the SGC cell lines.

Analysis of copy number variants

Ten gene amplifications and ten gene deletions in the six SGC cell lines were determined by CNVkit (**Figure 1B**). The highest frequency of gene amplification was *MYC*, whereas *FGFR2* amplification was observed in OCUM-2M, OCUM-14, as reported (14,28). *MET* amplification and *IGF1R* amplification were detected in OCUM-9 and OCUM-8, as reported (29). *KRAS* amplification was observed in OCUM-12. Genetic deletion was detected in nine tumor suppresser genes (**Figure 1B**).

STK11/LKB1 mutation

All *STK11/LKB1* mutations were verified by conventional Sanger capillary sequencing (**Figure 2A**). Nonsense mutation of *STK11/LKB1*was found at codon 253 (c.1874C>A) in OCUM-12 cells. Missense mutation of *STK11/LKB1* was found at codon 354 (c.2177C>G) in OCUM-2M cells and OCUM-14 cells. **Figure 2B** gives the locations and type of *STK11/LKB1* mutations detected in OCUM-12, OCUM-2M, and OCUM-14 cells. Nonsense mutation (Y253*) of *STK11/LKB1* was localized at the protein kinase domain in OCUM-12 cells. Missense mutation (F354L) of *STK11/LKB1* was localized at the low complexity of the gene in OCUM-2M and OCUM-14 cells, and was identified as one of the hotspot mutations by the COSMIC database. A three-dimensional model (PDB ID: 2WTK) indicated that Y253* is located on the surface of STK11/LKB1 protein with interaction to the heterotrimeric Lkb1-stradalpha-mo25alpha complex (**Figure 2C)**.

STK11/LKB1 expression and the effect of mTOR inhibitor on the proliferation of SGC cells.

Western blot analysis indicated that the expression of LKB1 was loss or reduced in OCUM-12 and OCUM-14 harboring *STK11/LKB1* mutation, whereas inconsistent results were detected in OCUM-2M. On the other hand, LKB1 remarkably expressed in OCUM-9 without *STK11/LKB1* mutation (**Figure 3A**). We found that Rapamycin significantly suppressed the growth of OCUM-12 and OCUM-14 in vitro, with IC50 values of approximately100 nmol/L, while Rapamycin had no effect in OCUM-2M and OCUM-9 (**Figure 3B**).

Clinicopathologic features of patients with gastric cancer according to STK11/LKB1 expression status

We analyzed 708 gastric patients according to STK11/LKB1 expression status using pLKB1 antibody and LKB1 antibody (**Table 1**). The pSTK11/LKB1 staining using pLKB1 antibody was mainly found at the nuclear and cytoplasm in cancer cells and normal cells (**Figure 3C**). The pSTK11/LKB1 expression was scored as 0, 1+, and 2+, as described above, and score of 0 or 1+ was identified as the pSTK11/LKB1-low, whereas score of 2+ was identified as the pSTK11/LKB1-high. To evaluate the significance of pSTK11/LKB1 expression in gastric cancer, we focused on 708 patients with gastric cancer to assess the correlations between clinicopathologic features and the pSTK11/LKB1 expression level. A total of 343 of the 708 gastric cancers were identified as having the low pSTK11/LKB1 expression status, and the remaining 365 patients were classified as having the high pSTK11/LKB1 expression status. **Table 1** summarizes the clinicopathological characteristics of the 708 patients according to their pSTK11/LKB1 expression status. Low pSTK11/LKB1

expression was significantly correlated with the following, compared to pSTK11/LKB1 high expression: macroscopic type 4 (22.7% vs. 4.9%, $p < 0.001$), histologic undifferentiated type (64.4% vs. 41.0%, $p < 0.001$), T status (63.0% vs. 40.2%, $p < 0.001$), nodal involvement (58.6% vs. 41.0%, $p < 0.001$), and lymphatic invasion (65.6% vs. 55.7%, $p = 0.009$), respectively. STK11/LKB1 expression status using LKB1 antibody demonstrated comparable correlations between clinicopathologic features and the STK11/LKB1 expression level (**Table 1**).

Survival

Figure 4A indicated the difference of the 5-year survival rate between the high- and low-expression groups at each stage. The 5-year survival of the patients with low pSTK11/LKB1 expression (48%) was significantly poorer than that of the patients with high pSTK11/LKB1 expression (68%) ($p < 0.001$, log-rank). In the patients at stage II, the 5-year survival of the patients with low pSTK11/LKB1 expression was significantly worse ($p = 0.021$, log-rank) compared to that of the patients with high pSTK11/LKB1 expression (**Figure 4B**). Across subgroup analyzed, low pSTK11/LKB1 expression was significantly associated with poor survival in patients with non-SGC ($p < 0.001$), whereas there were no associations in patients with SGC between pSTK11/LKB1 expression and overall survival **(Figure 4C).**

Table 2 summarizes the univariate and multivariate analysis with respect to overall survival outcomes based on pSTK11/LKB1 expression revealed by immunostaining in gastric cancer. A univariate analysis revealed that the low pSTK11/LKB1 expression status was a significant adverse prognostic factor ($p < 0.001$). After adjusting only for sex, histologic type, and nodal involvement, an association between the low pSTK11/LKB1 expression status and

survival outcome was still evident, with a close association noted after further adjustment for lympho-vascular invasion. This association was materially affected by adjusting for the T factor and macroscopic type.

DISCUSSION

The pathogenic mutation of *STK11/LKB1* was frequently detected in three of the six SGC cell lines in this study. It has been reported that somatic mutation of *STK11/LKB1* was found in some types of carcinomas, including lung cancer, gynecological cancer, pancreatic cancer, and gastric cancer (9,30-32). In gastric cancer, previous cohorts reported that *STK11/LKB1* mutation was rare (< 1%) in global gastric cancer (8,9), whereas Ichikawa H *et al.* (10) reported that *STK11/LKB1* mutation was identified as a relatively frequent alternation (3.9%) in Japanese. These findings indicate that *STK11/LKB1* mutation might be frequent in Japanese gastric cancer which contains high incidence of SGC (33). To the best of our knowledge, this is the first report to demonstrate the frequent *STK11/LKB1* mutations in sporadic SGC.

Another reason for our successful identification of *STK11/LKB1* mutations in SGC might be that SGC cell lines were used as tumor samples in the NGS analysis. It is difficult to obtain enough cancer cells from clinical SGC specimens because SGC histologically contains few cancer cells with abundant stromal cells (1), and this may result in a failure to determine the pathogenic variants by genomic analysis. SGC cell lines consist of only cancer cells, and we were thus able to identify *STK11/LKB1* mutations in SGC by an NGS analysis.

A germline mutation of *STK11/LKB1* was initially identified as the cause of Peutz-Jeghers syndrome, which is characterized by hereditary polyposis and a high risk for various types of tumors (17,18). *STK11/LKB1* has been recognized as a tumor suppressor gene(18,31). It was reported that most of the *STK11/LKB1* alterations in sporadic cancers were point mutations and deletions, resulting in a loss of expression and function (31,34,35). In the present study, a nonsense mutation (Y253*) of *STK11/LKB1* was found at the kinase domain, indicating a loss of function by western blot analysis. The other was a missense

mutation of *STK11/LKB1* (F354L) which is described as a hotspot mutation in the COSMIC database. Forcet *et al.* demonstrated that F354L in *STK11/LKB1* showed a disruption of cellular polarity (36), the result of which might support our results identified in OCUM-14. Interestingly, a germline mutation of *STK11/LKB1* (F354L) has been reported in hereditary diffuse gastric cancer (37). These findings might indicate that *STK11/LKB1* mutation is pathogenic and correlated with SGC. On the other hand, there is no association in OCUM-2M between F354L *STK11/LKB1* mutation and STK11/LKB1 inactivation. This result suggested that occult molecular signaling might upregulate STK11/LKB1 activation in OCUM-2M. A further study of how the level of STK11/LKB1 activation was determined should be conducted using OCUM-2M.

The significance of the loss of STK11/LKB1 in gastric cancer is still uncertain. It has been reported that low STK11/LKB1 expression was associated with poor prognosis of patients with gastric cancer (38-40) which was concordant with our results. In addition, we found that SGC was frequently correlated with low STK11/LKB1 expression. One of the reasons for the low expression of STK11/LKB1 might be due to *STK11/LKB1* mutation in SGC.

No difference of survival in 95 SGC patients was found between the low-expression of STK11/LKB1 and the high-expression of STK11/LKB1 (Figure 4C). On the other hand, in 308 GC patients at early stage of T1, the low expression of STK11/LKB1 was significantly (p = 0.011) correlated with histologic undifferentiated-type (data not shown), from which SGC has been reported to be arose (1,2,41). These findings might indicate that low STK11/LKB1 expression, as well as *STK11/LKB1* mutation, is closely associated with carcinogenesis of SGC, but not prognosis of patients with SGC.

It has been reported that STK11/LKB1 was reported to inhibit the growth of tumor cells by the activation of AMP-activated protein kinase/mTOR signaling (21,42). Metabolic rewiring was recently suggested to be a driver of tumorigenesis following STK11/LKB1 loss (35). STK11/LKB1 inactivation stimulates mTOR signaling, which is associated with tumor growth in cancer (20,21,43). The present study demonstrated that mTOR inhibitor decreased the proliferation activity of SGC cells with *STK11/LKB1* mutation. Fukamachi *et al.* also reported that mTOR inhibitors were useful for a subset of SGC cells *in vivo* (44). These findings indicated that mTOR inhibitor might be promising for the treatment of SGCs with *STK11/LKB1* mutation.

GRANITE-1 study, which evaluated mTOR inhibitor efficacy in patients with advanced gastric cancer, has reported that advanced gastric cancer patients were tend to be non-responders to mTOR inhibitor (45).However, forest plot of overall survival in subgroups analysis of GRANITE-1 study indicated that the risk ratio of diffuse-gastric cancer patients was low in compared to that of intestinal gastric cancer patients. These findings might suggest that patient with SGC, which is one of diffuse-gastric cancer, might receive a benefit for the treatment of mTOR inhibitor.

Transforming growth factor-beta (TGFβ)/Smad signaling was reported to be closely associated with SGC (46-48). Moren *et al.* stated that STK11/LKB1 might inhibit TGFβ signaling, which is potent inducer of the epithelial-mesenchymal transition process (49). These findings suggested that the STK11/LKB1 loss of function by pathogenic mutation might be associated with the activation of TGFβ/Smad signaling, resulting in the progression of SGC. We previously reported that the inhibition of TGFβ signaling was identified to suppress tumor growth of SGC cells (50). These findings suggested that the treatment strategy of inhibiting TGFβ signaling combined with mTOR inhibitor might lead to the

development of novel therapeutic approaches for patients with SGC harboring *STK11/LKB1* mutation.

In conclusion, *STK11/LKB1* was frequently mutated in SGC cell lines. The low STK11/LKB1 expression might be responsible for the progression of SGC. mTOR signaling by *STK11/LKB1* mutation might be a promising therapeutic target for patients with SGC.

Funding

KAKENHI (Grant-in-Aid for Scientific Research, Nos. 18H02883 to M.Y.)

Abbreviations

CNV copy number variant

GC gastric cancer

InDel insertion and deletion

mTOR mammalian target of rapamycin

NGS next-generation sequencing

SGC scirrhous-type gastric carcinoma

SNV single nucleotide variant

STK11/LKB1 Serine Threonine Kinase 11/Liver Kinase B1

Acknowledgements

Conflict of Interest Statement**:** None declared.

Reference

1. Yashiro, M., *et al.* (2010) Cancer-stromal interactions in scirrhous gastric carcinoma. *Cancer Microenviron*, **3**, 127-35.
2. Yashiro, M., *et al.* (2018) The significance of scirrhous gastric cancer cell lines: the molecular characterization using cell lines and mouse models. *Hum Cell*, **31**, 271-281.
3. Ikoma, N., *et al.* (2019) Linitis Plastica: a Distinct Type of Gastric Cancer. *J Gastrointest Surg*.
4. Terashima, M., *et al.* (2019) Randomized phase III trial of gastrectomy with or without neoadjuvant S-1 plus cisplatin for type 4 or large type 3 gastric cancer, the short-term safety and surgical results: Japan Clinical Oncology Group Study (JCOG0501). *Gastric Cancer*, **22**, 1044-1052.
5. Fushida, S., *et al.* (2018) Multidisciplinary therapy for scirrhous gastric cancer: a retrospective analysis and proposal o f new treatment strategy. *Cancer Management and Research*, **10**, 3833-3839.
6. Kinoshita, H., *et al.* (2015) Diffuse-type gastric cancer cells switch their driver pathways from FGFR2 signaling to SDF1/CXCR4 axis in hypoxic tumor microenvironments. *Carcinogenesis*, **36**, 1511-20.
7. Cai, H., *et al.* (2019) Mutational landscape of gastric cancer and clinical application of genomic profiling based on target next-generation sequencing. *Journal of Translational Medicine*, **17**, 12.
8. Cristescu, R., *et al.* (2015) Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nature Medicine*, **21**, 449.
9. Bass, A.J., *et al.* (2014) Comprehensive molecular characterization of gastric adenocarcinoma. *Nature*, **513**, 202-209.
10. Ichikawa, H., *et al.* (2017) Actionable gene-based classification toward precision medicine in gastric cancer. *Genome Med*, **9**, 93.
11. Katona, B.W., *et al.* (2017) Gastric Cancer Genomics: Advances and Future Directions. *Cellular and Molecular Gastroenterology and Hepatology*, **3**, 211-217.
12. Kakiuchi, M., *et al.* (2014) Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. *Nature Genetics*, **46**, 583-587.
13. Cho, S.J., *et al.* (2018) KMT2C Mutations in Diffuse-Type Gastric Adenocarcinoma Promote Epithelial-to-Mesenchymal Transition. *Clinical Cancer Research*, **24**, 6556-6569.
14. Okuno, T., *et al.* (2019) Establishment of a New Scirrhous Gastric Cancer Cell Line with FGFR2 Overexpression, OCUM-14. *Annals of Surgical Oncology*, **26**, 1093-1102.
15. Ishimoto, T., *et al.* (2017) Activation of Transforming Growth Factor Beta 1 Signaling in Gastric Cancer-associated Fibroblasts Increases Their Motility, via Expression of Rhomboid 5 Homolog 2, and Ability to Induce Invasiveness of Gastric Cancer Cells. *Gastroenterology*, **153**, 191-204.e16.
16. Yashiro, M., *et al.* (2016) Fibroblast growth factor receptor signaling as therapeutic targets in gastric cancer. *World J Gastroenterol*, **22**, 2415-23.
17. Hemminki, A., *et al.* (1998) A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature*, **391**, 184-7.
18. Kullmann, L., *et al.* (2018) Controlling the master-upstream regulation of the tumor suppressor LKB1. *Oncogene*, **37**, 3045-3057.

19. Giannakis, M., *et al.* (2016) Genomic Correlates of Immune-Cell Infiltrates in Colorectal Carcinoma. *Cell Rep*, **15**, 857-865.
20. Carretero, J., *et al.* (2007) Dysfunctional AMPK activity, signalling through mTOR and survival in response to energetic stress in LKB1-deficient lung cancer. *Oncogene*, **26**, 1616-1625.
21. Shaw, R.J. (2009) LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiol (Oxf)*, **196**, 65-80.
22. Yashiro, M., *et al.* (1996) Differential responses of scirrhous and well-differentiated gastric cancer cells to orthotopic fibroblasts. *Br J Cancer*, **74**, 1096-103.
23. Yashiro, M., *et al.* (1995) Establishment of two new scirrhous gastric cancer cell lines: analysis of factors associated with disseminated metastasis. *Br J Cancer*, **72**, 1200-10.
24. Takemura, S., *et al.* (2004) Novel models for human scirrhous gastric carcinoma in vivo. *Cancer Sci*, **95**, 893-900.
25. Kato, Y., *et al.* (2010) Establishment and characterization of a new hypoxia-resistant cancer cell line, OCUM-12/Hypo, derived from a scirrhous gastric carcinoma. *Br J Cancer*, **102**, 898-907.
26. Talevich, E., *et al.* (2016) CNVkit: Genome-Wide Copy Number Detection and Visualization from Targeted DNA Sequencing. *PLoS Comput Biol*, **12**, e1004873.
27. Kanda, Y. (2013) Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*, **48**, 452-8.
28. Kunii, K., *et al.* (2008) FGFR2-amplified gastric cancer cell lines require FGFR2 and Erbb3 signaling for growth and survival. *Cancer Res*, **68**, 2340-8.
29. Sai, E., *et al.* (2019) Identification of candidates for driver oncogenes in scirrhous-type gastric cancer cell lines. *Cancer Sci*.
30. Waddell, N., *et al.* (2015) Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature*, **518**, 495-501.
31. Matsumoto, S., *et al.* (2007) Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene*, **26**, 5911-5918.
32. Zieba, S., *et al.* (2018) Somatic mutation profiling of vulvar cancer: Exploring therapeutic targets. *Gynecologic Oncology*, **150**, 552-561.
33. Maruyama, K., *et al.* (2006) Gastric cancer treated in 1991 in Japan: data analysis of nationwide registry. *Gastric Cancer*, **9**, 51-66.
34. La Fleur, L., *et al.* (2019) Mutation patterns in a population-based non-small cell lung cancer cohort and prognostic impact of concomitant mutations in KRAS and TP53 or STK11. *Lung Cancer*, **130**, 50-58.
35. Kottakis, F., *et al.* (2016) LKB1 loss links serine metabolism to DNA methylation and tumorigenesis. *Nature*, **539**, 390-395.
36. Forcet, C., *et al.* (2005) Functional analysis of Peutz-Jeghers mutations reveals that the LKB1 C-terminal region exerts a crucial role in regulating both the AMPK pathway and the cell polarity. *Hum Mol Genet*, **14**, 1283-92.
37. Hansford, S., *et al.* (2015) Hereditary Diffuse Gastric Cancer Syndrome CDH1 Mutations and Beyond. *Jama Oncology*, **1**, 23-32.
38. Hu, M., *et al.* (2019) Decreased expression of LKB1 is associated with epithelial-mesenchymal transition and led to an unfavorable prognosis in gastric cancer. *Hum Pathol*, **83**, 133-139.
39. Sun, J., *et al.* (2016) Decreased Expression of Tumor-suppressor Gene LKB1 Correlates with Poor Prognosis in Human Gastric Cancer. *Anticancer Res*, **36**, 869-75.
40. Jiang, S., *et al.* (2016) Clinical significance and role of LKB1 in gastric cancer. *Mol Med Rep*, **13**, 249-56.
41. Jung, K., *et al.* (2016) Borrmann Type 4 Advanced Gastric Cancer: Focus on the

Development of Scirrhous Gastric Cancer. *Clinical endoscopy*, **49**, 336-345.
42. Nguyen, H.B., *et al.* (2013) LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. *Oncogene*, **32**, 4100-4109.
43. Korsse, S.E., *et al.* (2013) Targeting LKB1 signaling in cancer. *Biochim Biophys Acta*, **1835**, 194-210.
44. Fukamachi, H., *et al.* (2019) A subset of diffuse-type gastric cancer is susceptible to mTOR inhibitors and checkpoint inhibitors. *Journal of Experimental & Clinical Cancer Research*, **38**, 23.
45. Ohtsu, A., *et al.* (2013) Everolimus for Previously Treated Advanced Gastric Cancer: Results of the Randomized, Double-Blind, Phase III GRANITE-1 Study. *Journal of Clinical Oncology*, **31**, 3935-3943.
46. Shinto, O., *et al.* (2010) Inhibitory effect of a TGFbeta receptor type-I inhibitor, Ki26894, on invasiveness of scirrhous gastric cancer cells. *Br J Cancer*, **102**, 844-51.
47. Komuro, A., *et al.* (2009) Diffuse-type gastric carcinoma: progression, angiogenesis, and transforming growth factor beta signaling. *J Natl Cancer Inst*, **101**, 592-604.
48. Ehata, S., *et al.* (2011) Transforming growth factor-beta decreases the cancer-initiating cell population within diffuse-type gastric carcinoma cells. *Oncogene*, **30**, 1693-705.
49. Morén, A., *et al.* (2011) Negative regulation of TGFβ signaling by the kinase LKB1 and the scaffolding protein LIP1. *The Journal of biological chemistry*, **286**, 341-353.
50. Kawajiri, H., *et al.* (2008) A novel transforming growth factor beta receptor kinase inhibitor, A-77, prevents the peritoneal dissemination of scirrhous gastric carcinoma. *Clin Cancer Res*, **14**, 2850-60.

Table and Figure Legends

Table 1. Patient demographics in resected cases according to STK11/LKB1 expression status.

Table 2. Cox Proportional Hazards Analyses of time to death among 708 patients with pSTK11/LKB1 low expression.

Table 1. Patient demographics in resected cases according to STK11/LKB1 expression status

Variables		pSTK11/LKB1 expression*			STK11/LKB1 expression**		
		low	high	*p* value	low	high	*p* value
		n = 343 (%)	n = 365 (%)		n = 254 (%)	n = 454 (%)	
Age	Mean [range] years old	66 [57 - 72]†	67 [59 - 73]	0.194	68 [59 - 73]†	66 [57 - 73]	0.271
Sex							
	female	130 (37.9)	97 (26.5)	0.001	86 (33.9)	141 (31.1)	0.451
	male	213 (62.1)	268 (73.5)		168 (66.1)	313 (68.9)	
Macroscopic type							
	Scirrhous-type	78 (22.7)	18 (4.9)	< 0.001	51 (20.1)	45 (9.9)	< 0.001
	Other-types	265 (77.3)	347 (95.1)		203 (79.9)	409 (90.1)	
histologic type							
	differentiated	122 (35.6)	216 (59.0)	< 0.001	109 (42.9)	229 (50.4)	0.06
	undifferentiated	221 (64.4)	149 (41.0)		145 (57.1)	225 (49.6)	
T status							
	T1/2	127 (37.0)	219 (59.8)	< 0.001	91 (35.8)	255 (56.2)	< 0.001
	T3/4	216 (63.0)	146 (40.2)		163 (64.2)	199 (43.8)	
Nodal involvement							

	negative	142 (41.4)	215 (59.0)	< 0.001	102 (40.2)	255 (56.2)	< 0.001
	positive	201 (58.6)	150 (41.0)		152 (59.8)	199 (43.8)	
Stage	(UICC-7th ††)						
	I/II	160 (46.6)	255 (69.7)	< 0.001	121 (47.6)	294 (64.8)	< 0.001
	III/IV	183 (53.4)	110 (30.3)		133 (52.4)	160 (35.2)	
Lymphatic invasion							
	negative	118 (34.4)	161 (44.3)	0.009	80 (31.5)	199 (43.8)	0.001
	positive	225 (65.6)	204 (55.7)		174 (68.5)	255 (56.2)	
Venous invasion				0.914			0.284
	negative	275 (80.2)	290 (79.5)		197 (77.6)	368 (81.1)	
	positive	68 (19.8)	75 (20.5)		57 (22.4)	86 (18.9)	
STK11/LKB1 expression status**							
	low	178 (70.0)	76 (30.0)	< 0.001			
	high	165 (36.3)	289 (63.7)				

† Values are median [interquartile range]

†† The 7th version of Union for International Cancer Control classification

* Immunohistochemical staining using pLKB1 antibody

** Immunohistochemical staining using LKB1 antibody

Table 2. Cox Proportional Hazards Analyses of time to death among 708 patients with pSTK11/LKB1 low expression

Model	Hazard Ratio		*p* value
Unadjusted	1.85	(1.44-2.4)	<0.001
Adjusted for sex, histologic type and nodal involvement	1.33	(1.02-1.72)	0.03
Adjusted for sex, histologic type, nodal involvement and lympho-vascular invasion	1.34	(1.03-1.74)	0.03
Multivariable adjusted *	1.07	(0.81-1.40)	0.64

*Adjusted for sex, histologic type, nodal involvement, lympho-vascular invasion, T status and macroscopic type

Figure 1. Gene alterations in the scirrhous-type gastric cancer cell lines. A, Pathogenic alterations. A total of 33 mutated genes that harbored a point mutation, substitution, or copy number variation were detected in the six scirrhous-type gastric cancer cell lines. **B, Chromosome copy number variants in the SGC cell lines.** Ten gene amplifications (red arrows) and ten gene deletions (blue arrows) in the six SGC cell lines were determined by CNVkit (26). All amplification and deletion genes are listed according to the log2 ratio values, which were plotted by the relative genome order (|log2_ratio>1|).

Figure 2. *STK11/LKB1* mutations and protein structure. A, Sanger sequencing of *STK11/LKB1* in the six scirrhous-type gastric cancer cell lines. Nonsense mutation at codon 253 (c.1874C>A) was found in OCUM-12 cells. Missense mutation at codon 354 (c.2177C>G) was found in OCUM-2M cells and OCUM-14 cells. **B, Mutations Map on a liner protein of *STK11/LKB1* and its domains (lollipop plots).** Nonsense mutation Y253*, a mutation in codon 253 (Tyrosine → stop), was located on the protein kinase position in OCUM-12 cells. Missense mutation, a mutation in codon 354 (Phenylalanine → Leucine), was in the low complexity (*purple region*) in OCUM-2M cells and OCUM-14 cells. **C, Three-dimensional structure of STK11/LKB11.** Three-dimensional computational model showed STK11/LKB1 protein with the heterotrimeric Lkb1-stradalpha-mo25alpha complex. The nonsense mutation (Y253*) of *STK11/LKB1* (*arrow*) is located on the surface of the protein. Red lesion indicates the deletion protein by stop codon.

Figure 3. Correlation between STK11/LKB1 expression and mTOR signaling in SGC cell lines. A, The western blot analysis showed the expressions of LKB1 and β-actin in SGC cells. LKB1 was expressed in all SGC cell lines except for OCUM-12. β-actin was used

as a loading control. **B, Inhibition of mTOR signaling in SGC cell lines using Rapamycin.** Rapamycin inhibited the proliferation of two SGC cells (OCUM-12, OCUM-14) harboring *STK11/LKB1* mutation. In contrast, SGC cells with apparent expression of STK11/LKB1 were not sensitive to Rapamycin regardless of *STK11/LKB1* mutation. Graph show mean ± SD ($n = 6$). **$p < 0.01$ compared with control. **C, Representative picture of STK11/LKB1 expression.** 2+, STK11/LKB1 expression was mainly observed in nuclear and cytoplasm of cancer cells and normal cells using pLKB1 antibody, although it was mainly observed in cytoplasm of cancer cells using LKB1 antibody. 1+, weak staining of STK11/LKB1 was found in 50% of cancer cells. 0, weak staining of STK11/LKB1 was found in 5% of cancer cells.

Figure 4. The overall survival of 708 patients with gastric cancer according to their STK11/LKB1 expression status. **A, Overall survival of gastric cancer patients based on pSTK11/LKB1 expression.** The 5-year survival rate of the patients with low pSTK11/LKB1 expression was significantly poor compared to that of the patients with high expression (48% vs. 68%, $p < 0.001$). **B:** The Kaplan-Meier survival curve indicates that the overall survival of the gastric cancer patients with low pSTK11/LKB1 expression was significantly worse ($p = 0.021$) than that of the patients with high pSTK11/LKB1 expression at pStage II. **C, Overall survival of scirrhous or non-scirrhous gastric cancer patients according to pSTK11/LKB1 expression.** In patients with non-scirrhous-type gastric cancer, the survival of patients with low pSTK11/LKB1 expression was significantly poor ($p < 0.001$), while no significant difference of prognosis was found in patients with scirrhous-type gastric cancer. N.S. means “not significant”.

Figure 1. Nishimura S. *et al.*

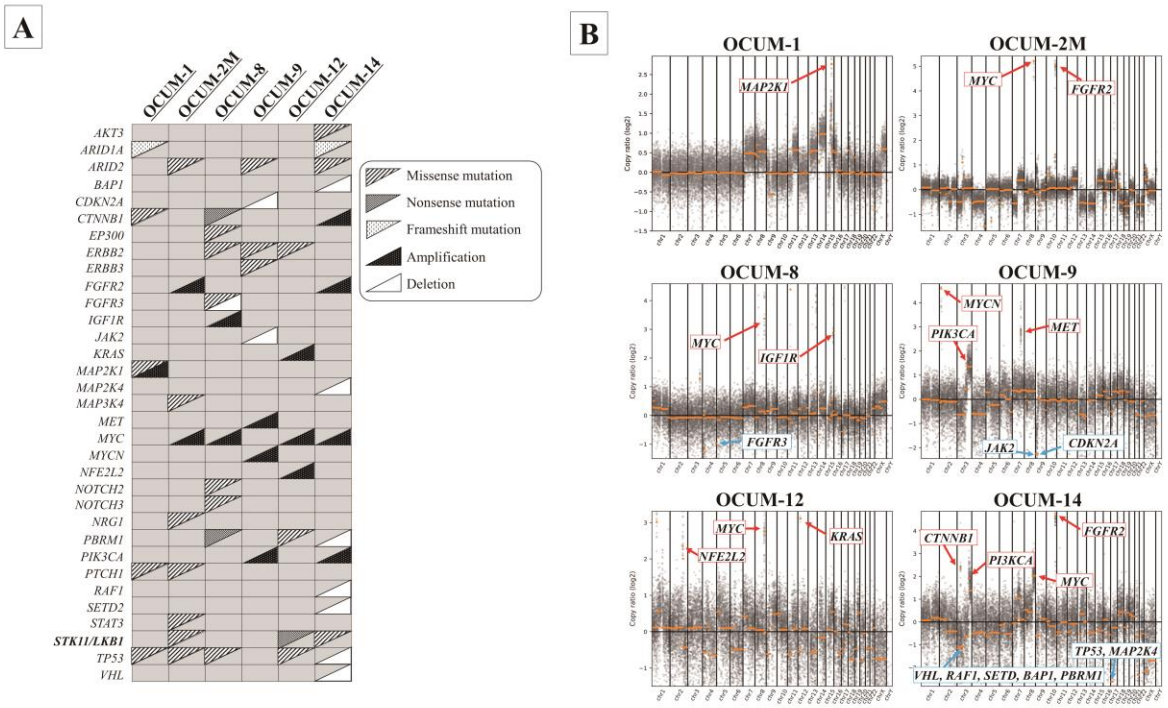

Figure 2. Nishimura S. *et al.*

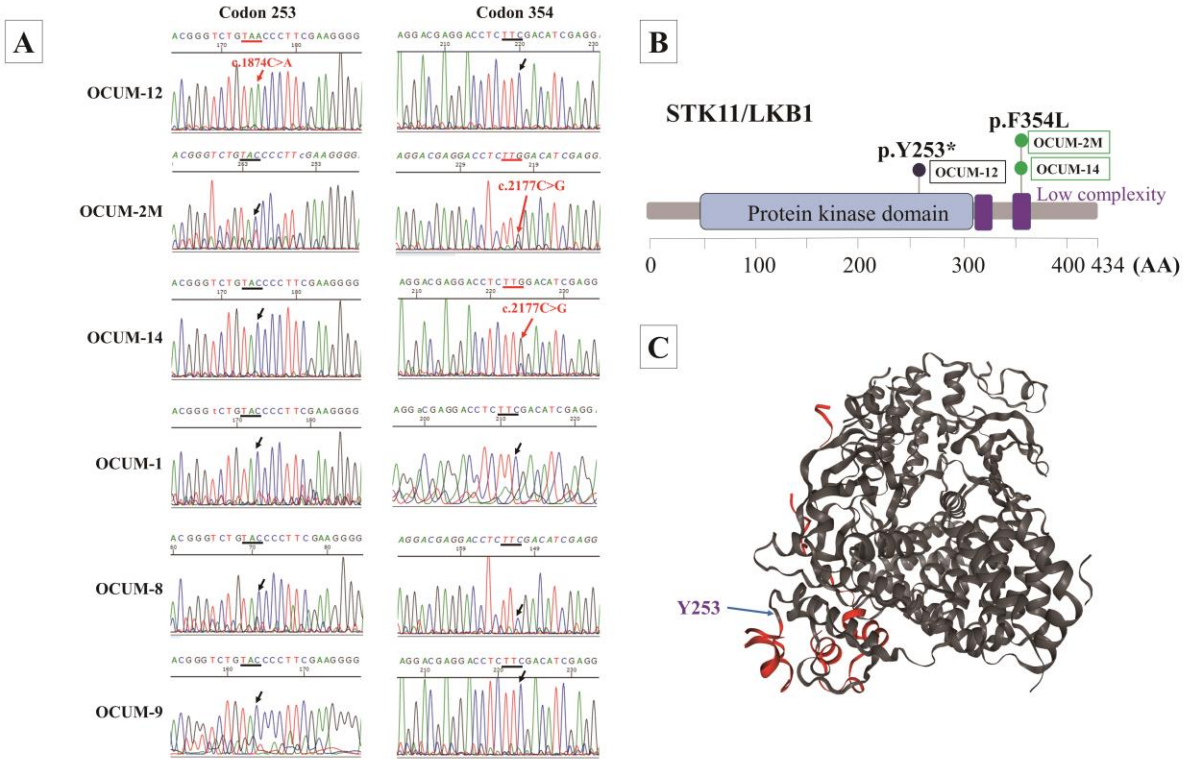

Figure 3. Nishimura S. *et al.*

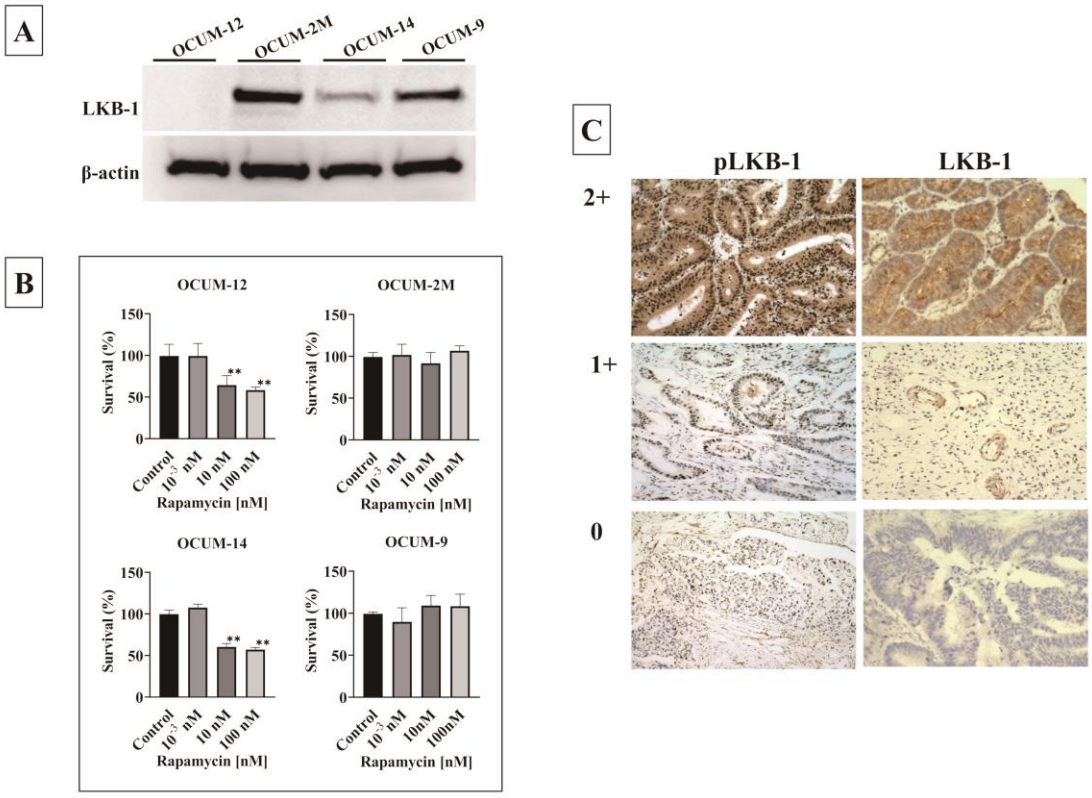

Figure 4. Nishimura S. *et al.*