HHS Public Access

Author manuscript

J Gastrointest Surg. Author manuscript; available in PMC 2022 September 01.

Published in final edited form as:

J Gastrointest Surg. 2021 September ; 25(9): 2231–2241. doi:10.1007/s11605-020-04889-9.

Activin A receptor type 2A mutation affects the tumor biology of microsatellite instability-high gastric cancer

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Abstract

Background—Activin A receptor type 2A (*ACVR2A*) is one of the most frequently mutated genes in microsatellite instability-high (MSI-H) gastric cancer. However, the clinical relevance of the *ACVR2A* mutation in MSI-H gastric cancer patients remains unclear. The aims of this

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Contributions

Specific contributions are as follows: conception and design of study, K. Yuza and M. Nagahashi; acquisition and analysis of clinical data, H. Ichikawa, T. Hanyu, T. Ishikawa, and J. Sakata; bioinformatics and analysis of genomic data, Y. Shimada and S. Okuda; completion of experiments, M. Nakajima, S. Takeuchi, Y. Matsuda, and M. Abe; drafting of the manuscript, K. Yuza and M. Nagahashi; supervision of the study, K. Sakimura, K. Takabe, and T. Wakai. All authors revised the manuscript critically for content. All authors approved the manuscript.

Conflict of interest

All authors declare that they have no competing interests.

study were to explore the effect of *ACVR2A* mutation on the tumor behavior and to identify the clinicopathological characteristics of gastric cancer patients with *ACVR2A* mutations.

Methods—An in vitro study was performed to investigate the biological role of ACVR2A via CRISPR/Cas9-mediated *ACVR2A* knockout MKN74 human gastric cancer cells. 124 patients with gastric cancer were retrospectively analyzed and relations between MSI status, *ACVR2A* mutations, and clinicopathological factors were evaluated.

Results—*ACVR2A* knockout cells showed less aggressive tumor biology than mock-transfected cells, displaying reduced proliferation, migration and invasion ($P < 0.05$). MSI mutations were found in 10% (13/124) of gastric cancer patients, and *ACVR2A* mutations were found in 8.1% (10/124) of patients. All *ACVR2A* mutations were accompanied by MSI. The 5-year overall survival rates of *ACVR2A* wild-type patients and *ACVR2A*-mutated patients were 57% and 90%, respectively ($P = 0.048$). Multivariate analysis revealed that older age ($P = 0.015$), distant metastasis ($P < 0.001$), and *ACVR2A* wild-type status ($P = 0.040$) were independent prognostic factors for overall survival.

Conclusions—Our study demonstrated that gastric cancer patients with *ACVR2A* mutation has a significantly better prognosis than those without. Dysfunction of ACVR2A in MKN74 human gastric cancer cells caused less aggressive tumor biology, indicating the importance of ACVR2A in the progression of MSI-H tumors.

Keywords

Activin Receptors; Stomach Neoplasms; Microsatellite Instability; Transforming Growth Factor-β; CRISPR-Cas Systems

Introduction

Gastric cancer (GC) is a highly lethal disease and is the third-leading cause of cancer-related death in the world, and its incidence is markedly high in Eastern Asia, including Japan.[1] The genomic complexity and tumor heterogeneity of GC are thought to be the main reasons for the poor prognosis,[2] despite the significant progress made in multidisciplinary treatment approaches incorporating surgery, radiotherapy, conventional chemotherapy, and newly developed molecular targeted therapies.[3] Therefore, a better understanding of the molecular and genetic characteristics and the underlying mechanisms is essential for achieving effective and personalized therapy for GC.

Recently, several groups have proposed new classification systems based on distinct molecular subtypes and genomic alterations in GC.[4, 5] Microsatellite instability-high (MSI-H) GC is an important subtype which is related to the loss of function of DNA mismatch repair genes. MSI status is associated with female sex, intestinal type, less aggressive cancer stages, and better survival compared with microsatellite stable (MSS) status and is characterized by elevated mutation rates, including mutations of genes encoding targetable oncogenic signaling proteins.[6] Genes that consist of mononucleotide repeats in their coding sequence, such as activin A receptor type 2A (*ACVR2A*), are potential candidates for frameshift mutation in MSI-H tumors.[7] ACVR2A is a transmembrane receptor with serine/threonine kinase activity, which transduces a signal when a ligand from the transforming

growth factor-β (TGF-β) superfamily binds to the extracellular domain. Activin and TGF-β are reported to have dual and opposing roles in carcinogenesis, also known as the molecular switch.[8] In early disease stages, they may function to suppress cancer growth, while in advanced disease stages, they may promote proliferation and migration, increasing metastatic behavior.[8]

Although numerous reports have focused on MSI-H colon cancers and their characteristics, the role of MSI in GCs remains uncertain. *ACVR2A* has been identified as one of the most frequently mutated genes in MSI-H tumors, and several studies have researched its biological function in carcinogenesis.[9, 10] However, clinical data in GC patients with *ACVR2A* mutations are limited, and the clinicopathological characteristics have not been thoroughly investigated. Moreover, the clinical relevance of patients with *ACVR2A* mutations associated with MSI is unclear, and the underlying mechanism has yet to be explored.

The aims of this study were to examine the effect of *ACVR2A* mutation on the tumor behavior, and to confirm the clinical characteristics of gastric cancer patients with *ACVR2A* mutations.

Materials and Methods

Cell lines and culture conditions

The human GC cell line MKN74 was purchased from the RIKEN Bioresource Center (Tsukuba, Ibaraki, Japan). Cells were maintained in 5% CO_2 at 37°C in RPMI-1640 medium (Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fischer Scientific Inc., Waltham, MA).

Establishment of *ACVR2A* knockout cells by the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system

CRISPR/Cas9-mediated *ACVR2A* knockout cells were established as previously described.[11] The following sequences were used for sgRNA synthesis and are identical to part of exon 4 of *ACVR2A*: 5′- TAG GGT GGC TTA GGT GTA AC −3′. Gene knockout was validated by sequencing genomic DNA from targeted cells.

Western blotting

Western blotting was performed as previously described.[12] Briefly, cells were lysed in buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (cOmplete; Roche Diagnostics, Basel, Switzerland). Protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). After samples were heat-denatured in Laemmli sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue), 10 μg of protein was separated in 4–15% or 10% SDS–PAGE gels and transferred onto Immobilon-P polyvinylidene difluoride membranes. After blocking for 1 hour in 5% skim milk, the membranes were probed with appropriate primary antibodies overnight at 4°C. Finally, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse

or anti-rabbit secondary antibodies for 1 hour. Signals were detected with an Enhanced Chemiluminescence Kit (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Image analysis software (ImageJ, ver. 1.44; NIH, Bethesda, MD) was used to quantify band intensities, and values were normalized to β-actin levels. The antibodies used were anti-ACVR2A (#ab135634; Abcam, Cambridge, MA, USA), anti-phospho-Smad2 (#3108; Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (#A5441; Sigma–Aldrich, St. Louis, MO, USA).

Proliferation assay

Cell proliferation was measured by using a Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindo Laboratories Co., Ltd., Kumamoto). Briefly, 5,000 cells were seeded in 96-well plates. After preincubation for 24 hours, WST-8/CCK-8 solution was added to the test cells. Cell viability was assessed at an absorbance of 450 nm as determined by a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA) at 1 hour and 26 hours after preincubation.

Scratch assay

Scratch assays were performed according to the protocol of Liang *et al.*[13] Briefly, cells were seeded on 12-well plates at a density of 5×10^5 cells per well and grown to confluence. After uniformly scratching the cell layer with a sterile 200-μl pipette tip, the cell debris was washed away with PBS, and this time was considered 0 hours. Images were captured at the same site at 0 hours and 63 hours after scratching to observe the process of wound healing. The areas of wound closure were measured by ImageJ (ver. 1.51j8; NIH, Bethesda, MD).

Cell migration and invasion assay

Cell migration and invasion assays were performed according to the manufacturer's protocol (Cell Biolabs, San Diego, CA, USA). Briefly, serum-free RPMI containing 3.0×10^5 cells was placed on 8.0-μm-pore size membrane inserts in 24-well plates, and RPMI with 10% FBS was placed in the lower wells. After 48 hours, the cells remaining in the top chamber were carefully removed from the upper surface of the filter using a cotton swab. Cells that had migrated to the underside of the membrane were stained by Cell Stain Solution (Cell Biolabs). The cells were quantified at OD 560 nm after extraction from the membrane by Extraction Solution (Cell Biolabs).

Human GC tissue samples and gene evaluation

Previously we performed genomic sequencing of 207 Japanese GCs using 435-gene panel.[14, 15] Of the 207 patients, we enrolled 124 patients whose clinical data were efficient to evaluate and confirm the clinical characteristics. The 124 patients had undergone surgery at Niigata University Medical and Dental Hospital or Niigata Cancer Center Hospital from June 2009 to January 2016. The Institutional Review Boards of Niigata University (#771) and Niigata Cancer Center Hospital (#641) approved this study and the methods. The detailed method for gene evaluation has been described previously.[14] In brief, DNA extracted from formalin-fixed and paraffin embedded specimens was sequenced by CANCERPLEX (KEW Inc.; Cambridge, MA, USA), a comprehensive next-generation

sequencing-based analytical platform. Tumor mutation burden (overall mutational rate), DNA mismatch repair gene mutations and MSI status (MSI-H or MSS) were evaluated.

Neoadjuvant chemotherapy was administered to 15 of 124 patients (12%) and all of them were treated with docetaxel, cisplatin and fluorouracil combination regimen. Twelve patients (9.7%) with Stage IV disease received preoperative chemotherapy, 9 of whom underwent conversion surgery with curative intent, and 3 underwent palliative surgery. Ninety-nine patients underwent microscopically margin-negative surgery (R0 resection). Of the patients who underwent R0 resection, adjuvant chemotherapy was administered to 75 patients (76%), which most of them (72 patients) received S-1 alone or in combination with other agents. Twenty patients who underwent surgery with positive margins were assigned to systemic chemotherapy, and regimens such as S-1 alone or S-1 with docetaxel/cisplatin were employed. None of the patients received adjuvant immunotherapy. The indication and the regimen of these chemotherapies were determined by the discretion of the attending surgeon.

Statistical analyses

Medical records were obtained for all 124 patients. The cause of death was determined from the medical records, and the follow-up period was defined as the interval between the resection and the last follow-up. The cumulative incidence of overall survival was estimated using the Kaplan-Meier method, and differences in the incidence of events were evaluated using the log-rank test. Categorical variables were compared by Fisher's exact test, and continuous variables among the groups were analyzed by the Mann-Whitney U-test. The Cox proportional hazard model was applied to the multivariate analyses to identify the factors associated with overall survival. Statistical evaluations were performed using either GraphPad Prism (version 7.02 for Windows, GraphPad Software, Inc., La Jolla, CA, USA) or the Japanese version of SPSS Statistics software package (version 24.0, IBM Japan, Tokyo, Japan). All tests were two-tailed, and $P < 0.05$ was considered significant.

Results

ACVR2A knockout cells show less aggressive tumor biology than mock-transfected cells

We examined the biological roles of ACVR2A in GC utilizing CRISPR/Cas9-mediated MKN74-*ACVR2A* knockout cells. Western blotting showed that ACVR2A was detectable in the original parental cells and mock-transfected cells but undetectable in *ACVR2A* knockout cells (Fig. 1a). The phosphorylation levels of Smad2, a downstream signaling molecule in the TGF-β pathway, were then examined in *ACVR2A* knockout cells. When cells were stimulated with serum, the phosphorylation levels of Smad2 were decreased in *ACVR2A* knockout cells, with a 1.2-fold increase in mock-transfected cells and a 0.6-fold increase in *ACVR2A* knockout cells, compared to the original parental cells ($P < 0.05$; Fig. 1b). This confirmed that the activin signaling pathway was suppressed by *ACVR2A* knockout. We observed no apparent morphological changes in mock-transfected cells and *ACVR2A* knockout cells under phase-contrast microscopy (Fig. 1c).

The effect of ACVR2A on the proliferation of human GC cells was examined using a WST-8 assay. *ACVR2A* knockout cells proliferated significantly less than mock-transfected cells (Fig. 2a and 2b; $P = 0.026$). Next, we analyzed the effects of ACVR2A on the migratory and invasive behavior of GC cell lines. In the scratch assay, the scratched area in *ACVR2A* knockout cell cultures was significantly larger than that in mock-transfected cell cultures, indicating that *ACVR2A* knockout cells had reduced migration (Fig. 2c and 2d; $P < 0.001$). Furthermore, in the migration and invasion assay, *ACVR2A* knockout cells displayed less migration and invasion than mock-transfected cells (Fig. 2e and 2f; $P = 0.029$). These data suggest that ACVR2A plays an important role in the proliferation, migration, and invasion of GC cells.

MSI status in patients with GC

124 gastric cancer patients were evaluated and 13 (10%) were MSI-H and 111 patients (90%) were MSS. The mutation rate of MSI-H tumors (35 mutations/Mb, range 19–102, standard deviation 17.4) was significantly higher than that of MSS tumors (14 mutations/Mb, range 4–42, standard deviation 6.8; $P < 0.001$). Mutations in mismatch repair genes were found in 6 of 13 (46%) MSI-H patients (*MLH1* in one, *MSH6* in two, *PMS2* in two, and both *MSH6* and *PMS2* in one patient).

ACVR2A mutation was frequently observed in GC patients with MSI-H

ACVR2A mutation was found in 8.1% (10/124) of GC patients. Importantly, 77% (10/13) of MSI-H GCs had an *ACVR2A* mutation, compared to 0% (0/111) of MSS GCs ($P < 0.001$; Table 1), indicating a strong association between *ACVR2A* mutation and MSI status in GC patients. The *ACVR2A* gene consists of 11 exons, and exon 3 and exon 10 contain 8-bp polyadenine [(A8)] tracts that frequently have frameshift mutations in GC.[7, 16] All 10 patients with an *ACVR2A* mutation had a frameshift mutation in these polyadenine tracts, 90% (9/10) in exon 10, and 10% (1/10) in exon 3 (Supplemental Table 1). The mutation rates for GC patients with an *ACVR2A* mutation were significantly higher than those with wild-type *ACVR2A* ($P < 0.001$; Fig. 3a, insert). The MSI status, *ACVR2A* mutation status, and mutation rate of each of the 124 GC patients are shown in Fig. 3a.

Clinicopathological characteristics of patients with *ACVR2A* mutations

We next examined the clinicopathological characteristics of the 10 GC patients with an *ACVR2A* mutation compared with those of the 114 GC patients with wild-type *ACVR2A* (Table 1). Tumor located at the lower third of the stomach were more common ($P = 0.044$) in the *ACVR2A*-mutated group. There were no significant differences in the other characteristics between the two groups, including sex, age, Lauren classification, tumor size, lymph node metastasis, or pathological stage (Table 1).

ACVR2A mutation is associated with alterations of mismatch repair genes

Based on the strong association between the *ACVR2A* genotype and MSI status, we next examined whether the *ACVR2A* mutation was associated with alterations in mismatch repair genes. As shown in Table 2, the *PMS2* mutation was significantly associated with the *ACVR2A* mutation ($P < 0.001$). Mutations in other mismatch repair genes, such as

MLH1 and *MSH6*, were not significantly associated with *ACVR2A* mutation (Table 2). As ACVR2A is involved in the TGF-β signaling pathway,[17] we also examined the association between *ACVR2A* alterations and *SMAD4* and *TGFBR2*, which are members of the TGF-β signaling pathway; however, no association was observed (Table 2).

Survival

The median follow-up period was 68 (range 49–129) months.—The 5-year overall survival rates were 57% in the *ACVR2A* wild-type group and 90% in the *ACVR2A* mutated group ($P = 0.048$, Fig. 3b). Only 1 of 10 patients with an *ACVR2A* mutation died, who had advanced disease with peritoneal dissemination but underwent palliative surgery to control anemia and pyloric stenosis. The univariate analysis identified age, tumor size, depth of tumor invasion, lymph node metastasis, distant metastasis, and *ACVR2A* mutation status as significant prognostic factors associated with overall survival (Table 3). In the multivariate analysis with these six variables, older age ($P = 0.015$), distant metastasis ($P < 0.001$), and *ACVR2A* wild type status ($P = 0.040$) were independent prognostic factors of overall survival (Table 3). The 5-year disease specific survival rates after resection was 64% in the *ACVR2A* wild-type group and 90% in the *ACVR2A* mutated group ($P = 0.099$; Supplemental Fig. 1). Among the *ACVR2A* mutated patients who are still alive, 1 patient was suspected of recurrence in the paraaortic lymph nodes 3 months after the surgery. This patient is now followed-up without apparent disease, after treated by combination chemotherapy of S-1, cisplatin, and trastuzumab for 2 years which showed good response and the lymph nodes eventually shrunk.

Discussion

This study investigated the effect of *ACVR2A* mutation on GC *in vitro*, and studied the clinical relevance of *ACVR2A* mutation in Japanese GC patients. Utilizing our newly established *ACVR2A* knockout GC cells, we found that the *ACVR2A* mutation causes less aggressive tumor biology. *ACVR2A* mutations existed among MSI-H tumors, consistent with previous reports.[18] Among GC patients with an *ACVR2A* mutation, patients with distally located tumors predominated. These patients also have a better prognosis than GC patients with wild-type *ACVR2A.*

Past reports have indicated a strong association between MSI status and *ACVR2A* mutation in gastrointestinal cancers, including GCs.[7, 16, 19, 20] A study from Singapore reported that *ACVR2A* was mutated in 86% of MSI-positive GC tumors,[18] and our data are compatible with these reports, showing a 77% *ACVR2A* mutation in MSI-H GCs. In addition to the high frequency of mutation, the complete loss of ACVR2A protein expression caused by *ACVR2A* frameshift mutations has been reported in MSI-H colon cancers.[10] Taken together, disruption of the activin signaling pathway due to mutation of *ACVR2A* highlights the important role of ACVR2A in MSI-H tumors. Although the *ACVR2A* mutation in MSI-H tumors has been described in several studies, its clinical relevance has been scarcely reported. In colon cancer, the correlation of *ACVR2A* mutations with poor tumor grade and larger tumor size was shown, but no survival benefit was observed.[21] Our study demonstrated that *ACVR2A*-mutated patients have a significantly better prognosis than

those with wild-type *ACVR2A*. Therefore, *ACVR2A* mutation may in part contribute to the characteristics of MSI-H tumors; however, further studies with a larger number of patients are needed to clarify this.

ACVR2A is a member of the TGF-β receptor family that is involved in the induction of differentiation, growth suppression, homeostasis, apoptosis, and many other functions.[17] TGF-β signaling is also known to play an important role in epithelial-to-mesenchymal transition (EMT), a process that cancer cells undergo during disease progression.[22] While TGF-β-induced EMT has been well studied, there are only some conflicting reports on activin signaling and EMT. Some data suggest that activin promotes EMT,[23] while others suggest that activin does not lead to EMT.[24] In this study, we explored the mechanism of ACVR2A signaling in GC by utilizing *ACVR2A* knockout GC cells and showed that *ACVR2A* knockout cells were less proliferative and migrated less than *ACVR2A* mock-transfected cells. These results suggest that dysfunction of ACVR2A leads cells to the opposite of EMT or mesenchymal-to-epithelial transition (Fig. 3c). We were somewhat surprised to find decreased proliferation in *ACVR2A* knockout cells, given that activin signaling has been reported as growth suppressive, and cell proliferation was expected to increase in *ACVR2A* knockout cells.[9] Considering the complex signal crosstalk in TGF-β signaling, with multiple ligands and various downstream molecules interacting with ACVR2A, the pathway regulating cell proliferation may not be so simple. Nevertheless, taken together, our experimental data describing less aggressive tumor biology in *ACVR2A* knockout cells may provide clues to explain the significantly better prognosis in *ACVR2A*-mutated GC patients. Further investigations are required to examine this.

MSI-H is now recognized as a specific GC subtype. Although the effect of cytotoxic agents in MSI-H patients remains controversial,[25, 26] they are known to respond well to immune checkpoint inhibitor therapy.[27] Based upon our finding that *ACVR2A* mutation is highly associated with MSI-H, we cannot help but speculate that *ACVR2A* mutated patients with advanced GC may respond well to immunotherapy as well. Although this notion needs to be confirmed by the patient cohort that undergo such treatment, *ACVR2A* mutation may emerge as a potential predictive biomarker of immune checkpoint inhibitor therapy for GC patients, where patient selection is the biggest challenge in this modality.

The major limitation of this study is that it is a retrospective study with a limited number of patients and a short follow-up period for some patients. Further studies with a larger number of patients with *ACVR2A* mutations would help clarify the additional clinical characteristics. To our knowledge, however, this is one of the largest studies investigating the association between MSI and *ACVR2A* mutation in GC patients, and in spite of its limitations, this study offers insight into the biology of MSI-H GC.

Conclusion

In conclusion, to the best of our knowledge, this is the first study to report the *ACVR2A* mutation rate in Japanese GC patients and the clinicopathological characteristics of GC patients with *ACVR2A* mutations, including clinical outcomes. Dysfunction of ACVR2A in GC cells results in a less aggressive tumor biology, which could explain the trend of better

prognosis in MSI-H GC patients. Further basic and clinical studies are needed to investigate the biological mechanisms associated with *ACVR2A* mutations and the role of *ACVR2A* as a distinct therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to acknowledge founding from the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, Grant Numbers 17K10579 for K. Yuza, 19H03714 and 18K19576 for M. Nagahashi, 19K09212 for H. Ichikawa, 19K09165 for T. Hanyu, 17K10692 for M. Nakajima, 18K08612 for Y. Shimada, 17H04093 for T. Ishikawa, 18H04123 for S. Okuda, and 19K22651 for T. Wakai. M. Nagahashi is also supported by Uehara Memorial Foundation, and Tsukada Medical Foundation. K. Takabeis supported by NIH/NCI grant R01CA160688. The genomic sequencing in this project was supported by funding from Denka Co., Ltd.

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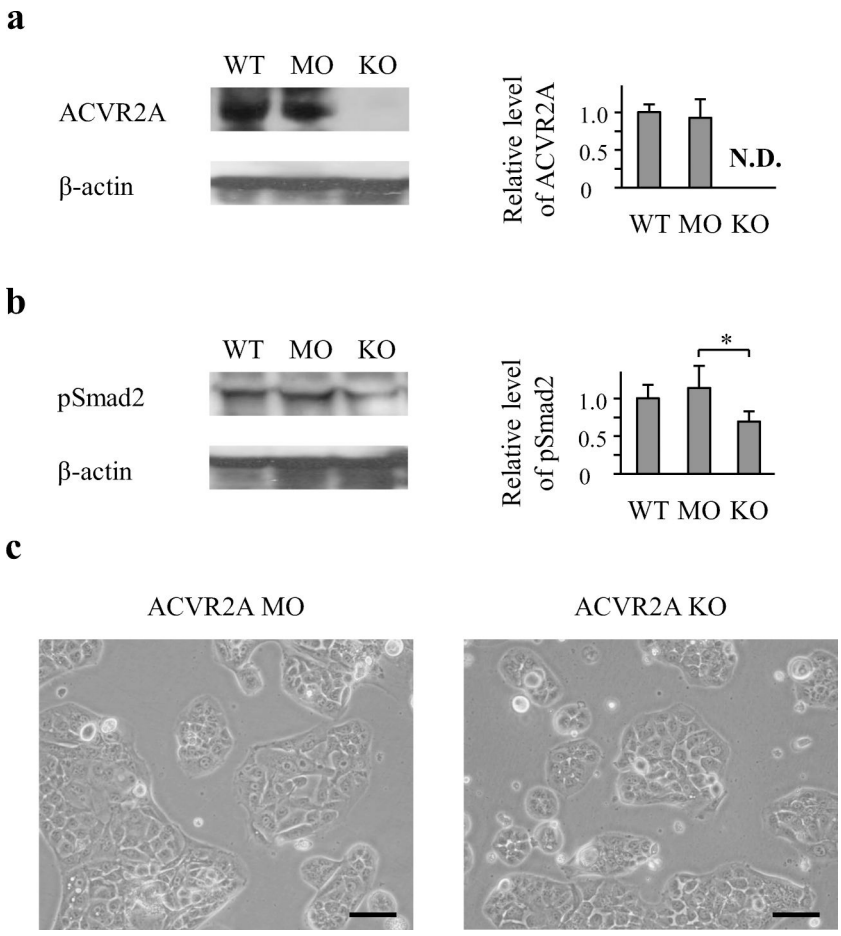

Fig. 1. Establishment of the *ACVR2A* knockout MKN74 cell line by the clustered regularly interspaced short palindromic repeats (CRISPR)-associated proteins 9 (Cas9) system.

a) Levels of ACVR2A in MKN74 cells were determined by Western blotting. **b)** Western blotting results and quantification of pSmad2 levels. Cells were serum-starved overnight and stimulated with culture media containing 10% fetal bovine serum for 30 min. Experiments were performed in triplicate, and band intensities were normalized to β-actin levels and expressed as fold change relative to original parental cells. Error bars represent SD (*, $P < 0.05$, vs. mock-transfected cells; N. D, not detected). **c)** Phase-contrast micrographs of mock-transfected cells and *ACVR2A* knockout cells. Scale bar, 200 μm. KO, *ACVR2A* knockout cells; MO, mock-transfected cells; WT, original parental cells.

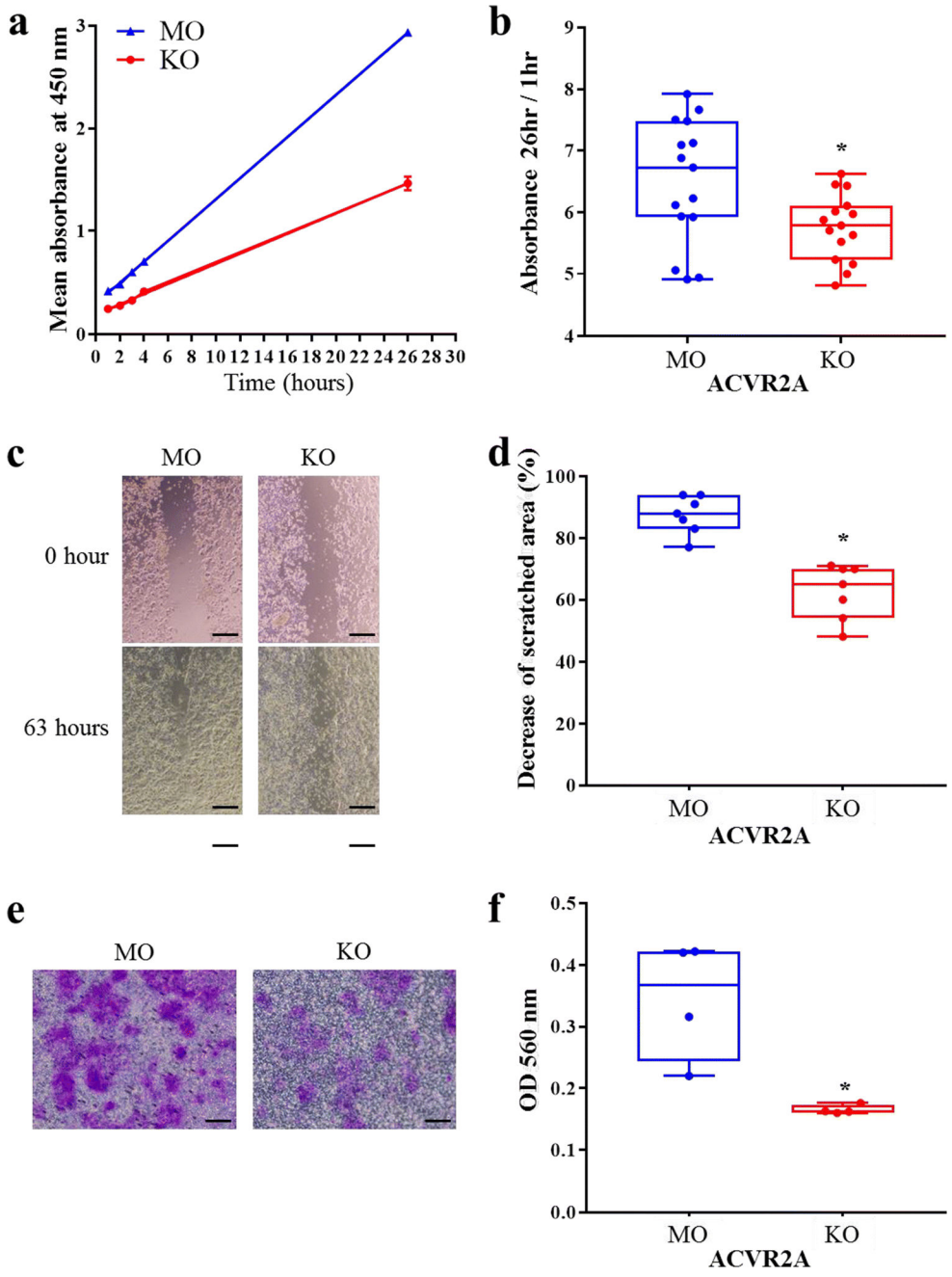

Fig. 2. Effect of *ACVR2A* knockout in gastric cancer cells.

a) and b) Cell proliferation was determined by WST-8 assay. The numbers of cells were determined by a microplate reader as optical density (OD). Data represent three independent experiments. The results are presented as the mean ± SD. **b)** Proliferation was calculated by comparing the OD 1 hour after the start of the experiment and the OD at 26 hours. *, $P = 0.026$. **c)** and **d)** Migratory ability of *ACVR2A* knockout cells in the scratch assay. **c)** Representative images from the results of the scratch assay in MKN74 cells. Scale bar, 200 μm. **d)** The decrease in the size of the scratched area was compared between *ACVR2A* knockout cells and mock-transfected cells. *, $P < 0.001$. **e) and f)** Invasion

ability of *ACVR2A* knockout cells. **e)** Representative images of migrated MKN74 cells after incubation for 48 hours and staining. Scale bar, 200 μm. **f)** The number of invaded cells through 8.0-μm pore-sized membranes was compared between *ACVR2A* knockout cells and mock-transfected cells as measured by absorbance at OD 560 nm. *, $P = 0.029$. KO, *ACVR2A* knockout cells; MO, mock-transfected cells.

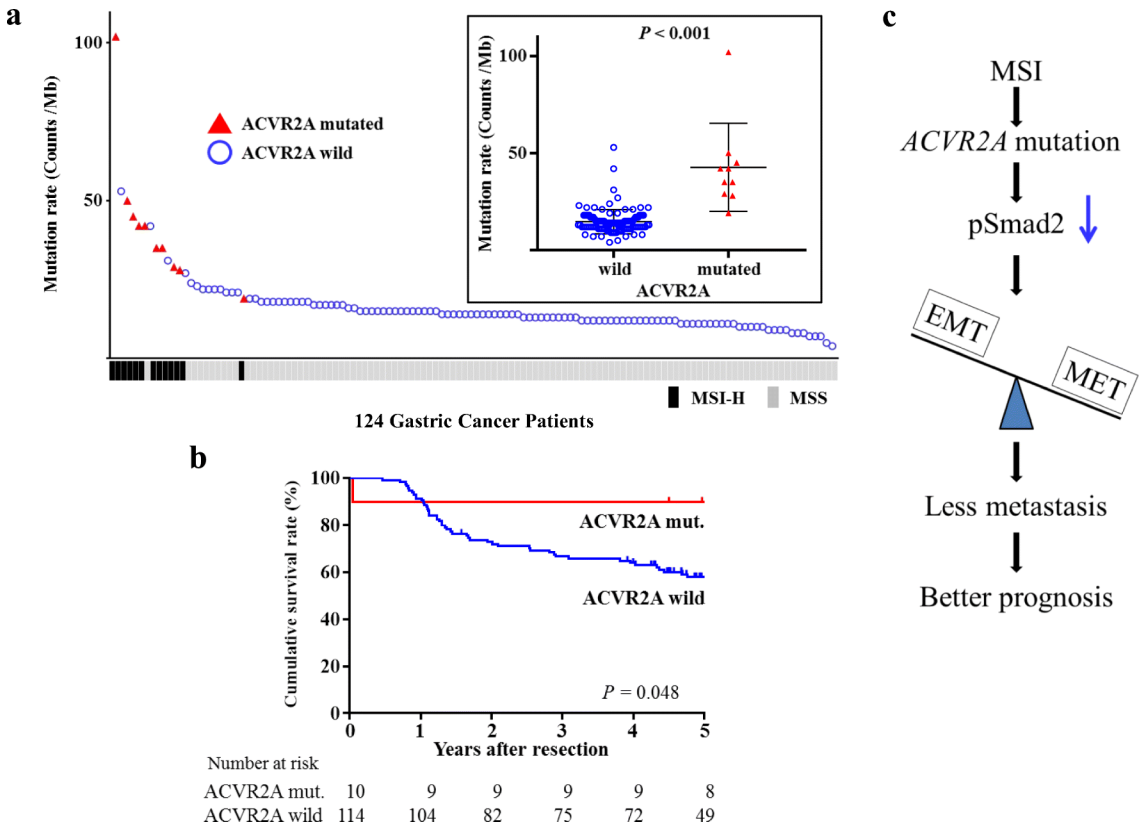

Fig. 3. *ACVR2A* mutation status and mutation rates in gastric cancer (GC), Kaplan-Meier survival estimates, and working hypothesis regarding how *ACVR2A* mutation affects the epithelial-mesenteric-transition process.

a) *ACVR2A* mutation status and mutation rates in the 124 tumor samples from GC patients. Each patient is shown with *ACVR2A* mutation status, mutation rate, and microsatellite instability (MSI) status. The patients are plotted in order of the mutation rate, starting from the patient with the highest mutation rate on the left. Black boxes, microsatellite instability-high (MSI-H); gray boxes, microsatellite stable (MSS); red triangles, *ACVR2A*-mutated patients; blue open circles, *ACVR2*A wild-type patients. Mutation rates in GC tissue from patients with wild-type *ACVR2A* (blue open circle) and those with an *ACVR2A* mutation (red triangle) are shown (insert); $P < 0.001$. **b)** Kaplan-Meier survival estimates of overall survival of 124 gastric cancer patients according to *ACVR2A* mutation. **c)** Microsatellite-unstable GC patients have a high frequency of *ACVR2A* mutations. Loss of ACVR2A results in downregulation of pSMAD2, a downstream effector of the canonical TGF-β pathway. While epithelial-mesenchymal transition (EMT) is an essential process for cancer cell metastasis, *ACVR2A* mutation may affect the process by downregulating TGF-β signaling and shifting the cancer cells from a mesenchymal-like phenotype to a more epithelial-like phenotype (mesenchymal-epithelial transition; MET). This would result in less dissemination and could explain the trend of a better prognosis in MSI-H gastric cancer patients.

Table 1

Clinicopathological characteristics according to the *ACVR2A* genotype in gastric cancer

Characteristic	*ACVR2A* WT (*n* = 114)	*ACVR2A* MT (*n* = 10)	*P* value
Microsatellite instability status, *n* (%)			< 0.001
MSS	111 (97)	0 (0)	
MSI-H	3 (3)	10 (100)	
Sex, *n* (%)			0.06
Male	84 (74)	4 (40)	
Female	30 (26)	6 (60)	
Median age, years (range)	67 (31–87)	72 (49–85)	0.29
Tumor location, *n* (%)			0.04
Lower	50 (44)	8 (80)	
Middle, upper	64 (56)	2 (20)	
Lauren classification, *n* (%)			0.51
Intestinal	51 (45)	4 (40)	
Diffuse/mixed	63 (55)	6 (60)	
Median tumor size, cm (range)	5.5 (1.4–20)	6.7 (3.2–11)	0.63
Macroscopic type, *n* (%)			> 0.99
Type 0, 1, 2	65 (57)	6 (60)	
Type 3, 4	49 (43)	4 (40)	
Depth of tumor invasion[a], *n* (%)			0.21
pT1, T2	24 (21)	0 (0)	
pT3, T4	90 (79)	10 (100)	
Lymph node metastasis, *n* (%)			0.14
Absent	29 (25)	5 (50)	
Present	85 (75)	5 (50)	
Distant metastasis, *n* (%)			> 0.99
Absent	93 (82)	8 (80)	
Present	21 (18)	2 (20)	
Stage[a], *n* (%)			> 0.99
I, IIA	31 (27)	2 (20)	
IIB, III, IV	83 (73)	8 (80)	
Lymphatic invasion, *n* (%)			0.73
Absent	32 (28)	2 (20)	
Present	82 (72)	8 (80)	
Venous invasion, *n* (%)			0.74
Absent	46 (40)	5 (50)	
Present	68 (60)	5 (50)	

[a]According to the Union for International Cancer Control 7th edition

MT, mutated; WT, wild-type; MSS, microsatellite stable; MSI-H, microsatellite instability-high

Table 2

Association between the *ACVR2A* genotype and mutations in mismatch repair genes or genes related to the transforming growth factor-β pathway

	ACVR2A		
Variable	**WT (*n* = 114)**	**MT (*n* = 10)**	***P* value**
MLH1			
WT	111	9	0.29
MT	3	1	
MSH6			
WT	110	8	0.074
MT	4	2	
PMS2			
WT	114	7	< 0.001
MT	0	3	
SMAD4			
WT	108	10	> 0.99
MT	6	0	
TGFBR2			
WT	110	9	0.35
MT	4	1	

MT, mutated; WT, wild-type

Table 3

Univariate and multivariate analyses of overall survival in 124 patients with gastric cancer

			Univariate	Multivariate	
Characteristic	**Number of patients**	**5-year survival rate (%)**	***P* value**	**Hazard ratio (95% CI)**	***P* value**
Sex			0.084		
Male	88	55			
Female	36	72			
Age (years)			0.004		
≤ 70	76	67		1.00	
> 70	48	47		1.95 (1.14–3.34)	0.015
Tumor location			0.295		
Lower	58	54			
Middle, upper	66	64			
Lauren classification			0.154		
Intestinal	67	54			
Diffuse/mixed	57	66			
Tumor size (cm)			0.021		
≤ 6.0	73	67			
> 6.0	51	48			
Depth of tumor invasion[a]			0.019		
pT1, T2	24	83		1.00	
pT3, T4	100	54		2.54 (1.00–6.43)	0.050
Lymph node metastasis			0.002		
Absent	34	79			
Present	90	52			
Distant metastasis			< 0.001		
Absent	23	68		1.00	
Present	101	18		3.43 (1.91–6.17)	< 0.001
Lymphatic invasion			0.086		
Absent	34	72			

Characteristic	Number of patients	5-year survival rate (%)	Univariate		Multivariate	
			P value	Hazard ratio (95% CI)		*P* value
Present	90	55				
Venous invasion			0.105			
Absent	51	66				
Present	73	55				
ACVR2A status			0.048			
Wild	114	57		1.00		
Mutated	10	90		0.13 (0.02–0.91)		0.040
Microsatellite instability status			0.135			
MSS	111	57				
MSI-H	13	77				

[a] According to the Union for International Cancer Control 7th edition

MSS, microsatellite stable; MSI-H, microsatellite instability-high; pT, pathological primary tumor classification