

## Clinical Significance of KIAA1199 as a Novel Target for Gastric Cancer Drug Therapy

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**Abstract.** *Background/Aim:* The KIAA1199 gene has been associated with cancer-cell proliferation, but its functions remain poorly studied. Here, we examined the clinical significance of the KIAA1199 mRNA levels in locally advanced gastric cancer (GC). *Materials and Methods/Results:* Using samples from 254 patients with stage II/III GC, we found significantly higher KIAA1199 levels in cancerous tissues compared to adjacent normal mucosa (ANM). There was no significant relationship between KIAA1199 expression and clinical features. Although overall survival rates (OSR) of patients, who underwent surgery did not correlate with KIAA1199 expression, patients who underwent adjuvant chemotherapy with S-1 and had high KIAA1199 levels displayed significantly lower OSR. KIAA1199 knock down (KIAA1199-KD) suppressed proliferation, invasiveness, and sensitivity of GC cells to 5-fluorouracil (5-FU). *Conclusion:* KIAA1199 expression appears to be a promising prognostic marker in patients with locally advanced GC, who underwent postoperative adjuvant chemotherapy with S-1. KIAA1199 may represent a novel target for GC pharmacotherapy.

Gastric cancer (GC) is the fifth most common cancer type (1,033,701 affected individuals in 2018) and accounts for the third highest number of cancer-related deaths (781,631 death cases in 2018) (1). In Japan, postoperative adjuvant S-1 chemotherapy is one of the standard treatments for locally advanced GC after radical resection (2-4). Fluorinated-pyrimidine is still the key drug for postoperative adjuvant chemotherapy against GC (5-7). However, such GC treatments are not sufficiently efficient (3, 6). Personalized postoperative adjuvant chemotherapy using biomarkers is a promising treatment strategy for improving outcomes of patients with locally advanced GC.

The KIAA1199 gene was discovered as an inner-ear-specific gene encoding the KIAA1199 protein (8). KIAA1199 is located at chromosome band 15q25.1, the same region where the brain tumor-suppressor gene was mapped (9-11). Recent studies have reported that KIAA1199 repression attenuates Wnt/ $\beta$ -catenin signaling and decreases the proliferation of colorectal cancer cells (12). Furthermore, our DNA microarray analysis showed a 10-fold increase in KIAA1199 expression in GC tissue compared to adjacent normal mucosa (ANM). Here, we examined the clinical significance of KIAA1199 expression in locally advanced GC and its potential as a new target for anti-GC agents.

### Materials and Methods

*Patients and samples.* The examined surgical samples of cancerous tissue and paired ANM were obtained from 254 patients with previously untreated stage II/III GC, who underwent radical

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resection. Tumor staging was evaluated using the 8th edition of the International Union Against Cancer TNM classification for GC. The patients underwent gastrectomy at Yokohama City University between January 2002 and July 2007. Informed consent was obtained from each patient and the ethics committees of Yokohama City University approved the protocol prior to the study (approval number: 18-7A-4). Samples were embedded in optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and immediately stored at  $-80^{\circ}\text{C}$ . None of the patients presented any other carcinomas.

**RNA extraction and complementary DNA (cDNA) synthesis.** Total RNA was extracted from GC tissue and ANM using TRIzol<sup>TM</sup> Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol and stored at  $-20^{\circ}\text{C}$ .

**Quantitative RT-PCR (qRT-PCR).** qRT-PCR was performed in a total volume of 15  $\mu\text{l}$  containing iQ SYBR-Green Supermix (Bio-Rad Laboratories), cDNA, and the following primers: sense primer 5'-GACCTCTCCATCCATCATAC-3' and antisense primer 5'-TTCCGTGAAGAAGCAGTG-3'. The oligonucleotide primers for the internal control  $\beta$ -actin were sense primer 5'-AGTTGCGTTACACCCTTCTTGAC-3' and antisense primer 5'-GCTCGCTCCAACCGACTGC-3'. The PCR program consisted of: initial denaturation (10 min at  $95^{\circ}\text{C}$ ); 40 cycles of cDNA denaturation (10 s at  $95^{\circ}\text{C}$ ), annealing (10 s at  $60^{\circ}\text{C}$  for KIAA1199 and at  $66^{\circ}\text{C}$  for  $\beta$ -actin), and primer extension (20 s at  $72^{\circ}\text{C}$ ); and final extension (10 min at  $72^{\circ}\text{C}$ ). Melting curves were analyzed to distinguish specific from nonspecific products and primer dimers. mRNA expression was evaluated by a standard curve consisted of three concentrations of  $\beta$ -actin cDNA (Clontech Laboratories, Inc., CA, USA). The average of triplicates of each measurement is presented.

**Immunohistochemical analysis.** Formalin-fixed and paraffin-embedded GC tissue sections were deparaffinized and soaked in 10 mM sodium citrate buffer at  $121^{\circ}\text{C}$  for 15 min to retrieve cell antigens. The sections were blocked and incubated overnight at  $4^{\circ}\text{C}$  with primary polyclonal anti-KIAA1199 antibody (Atlas Antibodies, Stockholm, Sweden) at 1:100 dilution. Peroxidase-labeled polymer (EnVision+HRP, rabbit, DAKO, Glostrup, Denmark) was used to detect antigen-antibody reaction. All sections were counterstained with hematoxylin. Samples were observed under an upright microscope (BX43; Olympus, Tokyo, Japan).

**MKN45 cell transfection with KIAA1199-targeting small-interfering RNAs (siRNAs).** Transient transfection of human GC cell line (MKN45) with KIAA1199-targeting siRNAs was performed as previously described (13). Briefly, MKN45 cells were plated at a density of  $1.0 \times 10^4$  cells/well in a micro-plate and the optimal siRNA concentration was determined. The cells were transfected using KIAA1199-targeting SMART pool siRNA (Dharmacon, Lafayette, CO, USA), Lipofectamine RNAiMAX (Invitrogen), and Opti-MEM I (Invitrogen). KIAA1199-KD was evaluated by qRT-PCR and western blotting. An optimal working concentration of siRNA was used to perform cell proliferation assays. All siRNA transfection experiments were performed three times.

**Western blotting.** Gastric cancer cells were washed twice with phosphate-buffered saline and lysed by incubation in Lysis A buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.0), 50 mM sodium chloride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 5 mM EDTA, 1 mM sodium orthovanadate, and a phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The proteins were resolved using SDS-PAGE and transferred onto a PVDF membrane (Invitrogen). After blocking, the membranes were exposed to anti-KIAA1199 (Abcam plc, Cambridge, UK) and anti- $\beta$ -actin primary antibodies (Abcam plc). The cells were then washed followed by incubation with goat anti-rabbit IgG H&L (HRP) secondary antibody (Abcam plc). Finally, the proteins were visualized using an ECL Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

**Cell proliferation and invasiveness assays.** Proliferation of KIAA1199-KD and control MKN45 cells was compared at 24, 48, and 96 h after plating  $1.0 \times 10^6$  cells/well in a microplate. Cell proliferation was assessed using the MTT-assay [3-(4, 5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, Roche] with a microplate reader (ImmunoMini NJ-2300, InterMed, Japan) with a readout at 540 nm. Cell invasiveness was compared at 6, 24, 48, and 72 h after plating  $1.0 \times 10^6$  cells/well in RPMI 1640 medium with no serum in the upper chamber of a Transwell insert (pore diameter, 8  $\mu\text{m}$ ; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was added to the bottom chamber. After 24 and 48 h, cells in the upper chamber were removed by scraping and the cells remaining in the lower chamber were stained with CyQuant GR dye (Chemicon) to assess the number of cells.

**Analyzing cell sensitivity to 5-FU.** KIAA1199-KD and control MKN45 cells were plated at  $1.0 \times 10^6$  cells/well and after 24 h they were treated with 5  $\mu\text{M}$  5-FU for 6, 24, 48, and 72 h. An MTT-assay was used to detect changes in 5-FU sensitivity of the cells.

**Statistical analysis.** Expression levels of KIAA1199 were compared between GC tissue and ANM using the Wilcoxon signed-rank test. The relationships between gene expression and clinical factors like age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, disease stage, lymphatic invasion, and venous invasion were evaluated using the  $\chi^2$  test. The postoperative OSR was analyzed using the Kaplan-Meier method and differences in OSR were assessed using the log-rank test. A Cox proportional-hazards regression model was used for univariate and multivariate analyses. Results of MTT and invasion assays were evaluated using the Mann-Whitney *U*-test. All statistical analyses were performed using IBM SPSS Statistics 18.0 (SPSS, Inc., Chicago, IL, USA). Two-sided *p*-values were calculated and a difference was considered significant when  $p < 0.05$ .

## Results

**Comparison of KIAA1199 expression between GC tissue and ANM.** KIAA1199 levels were significantly higher in cancer tissue than in ANM ( $p < 0.0001$ ; Figure 1).

**KIAA1199 expression in GC tissue.** Positive staining for KIAA1199 was observed in the cytoplasm and it was markedly more intense in GC cells than in stromal cells in both differentiated and undifferentiated GC types (Figure 2).

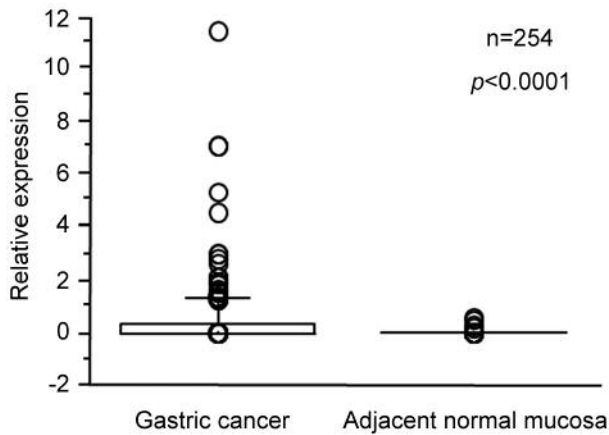


Figure 1. Comparison of KIAA1199 mRNA expression levels between GC tissue and ANM ( $p<0.0001$ ). Box boundaries indicate the 25th and 75th percentiles of the observed values and capped bars, the 10th and 90th percentiles. The solid line indicates the median.

**Relationship between KIAA1199 expression levels and clinical characteristics.** KIAA1199 expression levels were categorized as low or high according to the median value in all samples and the relationship with clinical characteristics is summarized in Table I. There was no significant correlation between KIAA1199 expression and any of the clinical features.

**Correlation between KIAA1199 expression levels and OSR.** Opposite correlation between OSR and KIAA1199 expression was observed in patients, who had received adjuvant chemotherapy with S-1. High expression of KIAA1199 was associated with significantly lower OSR ( $p=0.0015$ ; Figure 3A). However, OSR was not affected by the KIAA1199 levels in patients who underwent surgery alone (Figure 3B). The postoperative relapse-free survival was significantly lower in patients with high compared to low KIAA1199 levels ( $p=0.0047$ ; Figure 3C).

**Univariate and multivariate analyses of the relationships between clinical factors, KIAA1199 expression, and outcomes.** Univariate analysis revealed that serosal invasion and KIAA1199 expression were related to the outcomes. Multivariate Cox proportional hazards regression analysis showed that only KIAA1199 expression was related to the outcomes ( $p=0.0074$ ; Table II).

**Changes in invasiveness and proliferative activity in KIAA1199-KD GC cells.** KIAA1199-KD in GC cells suppressed their proliferation and invasiveness 24, 48, and 96 h after plating ( $p<0.05$ ; Figure 4A and B).

Table I. The relationship between KIAA1199 gene expression and clinical characteristics.

Variables/categories	KIAA1199 expression		p-Value
	Low (n=74)	High (n=73)	
Age	66.84±7.76	64.53±10.84	0.101
Gender			
Male	51	50	1
Female	23	23	
Size			
<6.5 cm	35	35	1
≥6.5 cm	39	38	
Histologic type			
Differentiated type	29	28	1
Undifferentiated type	45	45	
Depth of invasion			
T1	0	3	0.321
T2	12	9	
T3	17	18	
T4	45	43	
Stage			
II	24	19	0.469
III	50	54	
Lymph node metastasis			
Absent	12	8	0.471
Present	62	65	
Lymphatic invasion			
Absent	14	21	0.179
Present	60	52	
Venous invasion			
Absent	18	18	1
Present	56	55	

**Changes in sensitivity of KIAA1199-KD GC cells to 5-FU.** KIAA1199-KD GC cells appeared more sensitive to 5-FU and exhibited significantly decreased proliferation compared to control cells 6 h after 5-FU administration ( $p<0.05$ ). This effect was maintained until 96 h after 5-FU administration (Figure 4C).

## Discussion

The aim of this study was to reveal the clinical significance of KIAA1199 expression in locally advanced GC and its potential as a new target for anti-GC agents.

Several previous have reported that KIAA1199 mRNA expression is higher in colorectal (12), breast (14), and GC (15) cancer tissues than in normal tissues. Consistent with these findings, we observed higher KIAA1199 mRNA expression in GC tissue than in paired ANM.

It has been reported that increased KIAA1199 mRNA expression correlates with tumor metastasis nodes in non-small cell lung cancer (16). In a previous report, KIAA1199

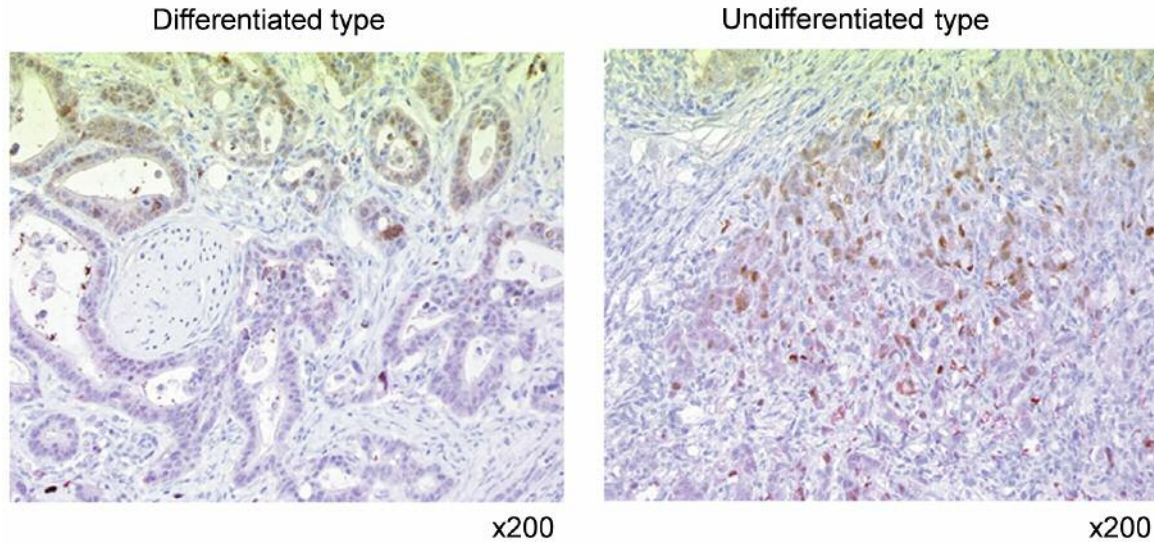


Figure 2. Immunohistochemistry of KIAA1199 protein, mainly located in the cytoplasm. Left panel shows the KIAA1199 staining in differentiated and the right in undifferentiated GC cells.

Table II. Univariate and multivariate Cox proportional hazards analyses of the relationship between survival, KIAA1199 gene expression, and clinical characteristics in the group that received S-1 adjuvant chemotherapy.

Variables/categories	n	Univariate analysis			Multivariate analysis		
		Hazard ratio	95%CI	p-Value	Hazard ratio	95%CI	p-Value
Age (years)							
<70	96	1		0.7688			
≥70	51	1.112	0.547-2.263				
Gender							
Female	46	1		0.6495			
Male	101	1.185	0.570-2.463				
Histological type							
Differentiated type	56	1		0.8994			
Undifferentiated type	91	1.052	0.514-2.155				
Tumor size, mm							
<65 mm	70	1		0.0922			
≥65 mm	77	1.795	0.990-3.546				
Serosa invasion							
Absent	59	1		<b>0.0458</b>	1	1.000-4.566	0.05
Present	88	2.169	1.014-4.630		2.137		
Lymph node metastasis							
Absent	20	1		0.3011			
Present	127	1.869	0.571-6.098				
Venous invasion							
Absent	111	1		0.1899			
Present	36	1.799	0.748-4.329				
Lymphatic invasion							
Absent	112	1		0.5287			
Present	35	1.304	0.571-2.976				
KIAA1199 gene expression							
High	73	1		<b>0.0068</b>	1		<b>0.0074</b>
Low	74	2.891	1.339-6.25		2.865	1.326-6.173	

CI: Confidence interval. Bold values indicate statistical significance at  $p < 0.05$ .

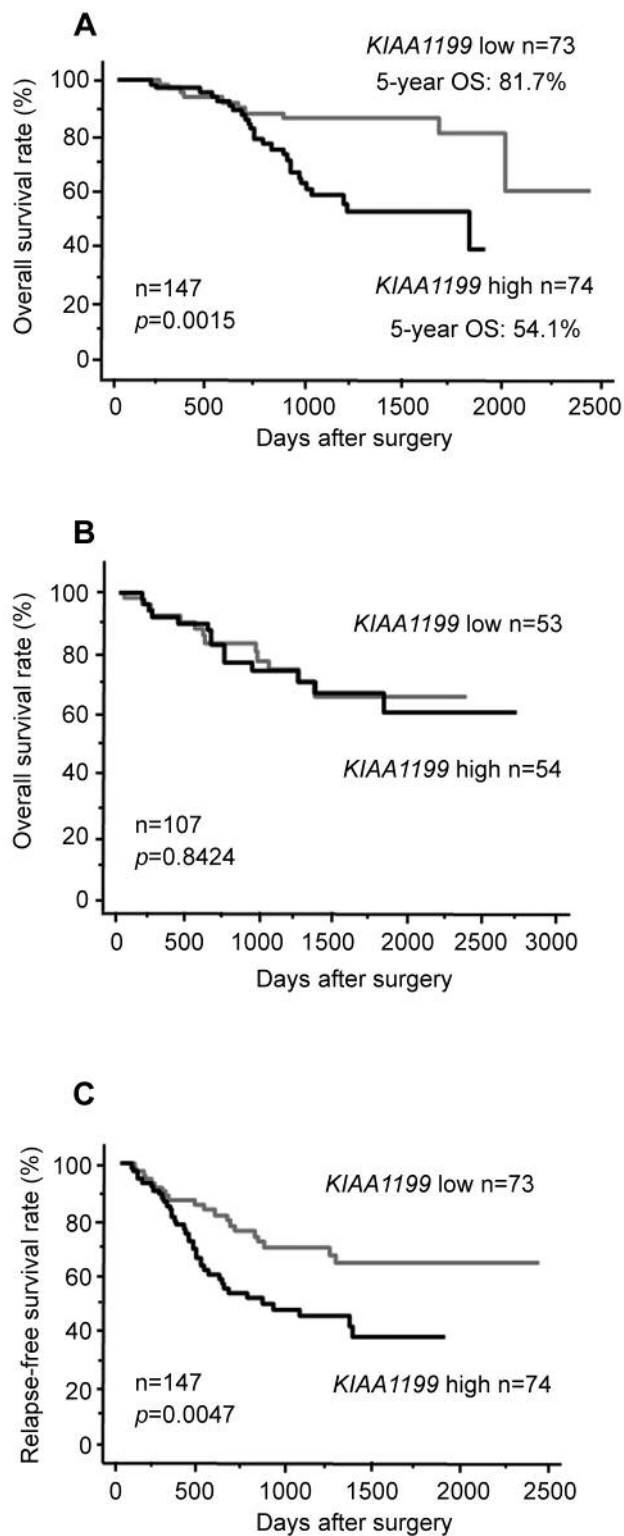


Figure 3. The relationship between KIAA1199 mRNA expression levels and cell survival in patients given adjuvant chemotherapy with S-1 ( $p=0.0015$ ) (A); in patients with surgery alone ( $p=0.8424$ ) (B), or in patients who received adjuvant chemotherapy with S-1 ( $p=0.0047$ ) (C). OS: Overall survival.

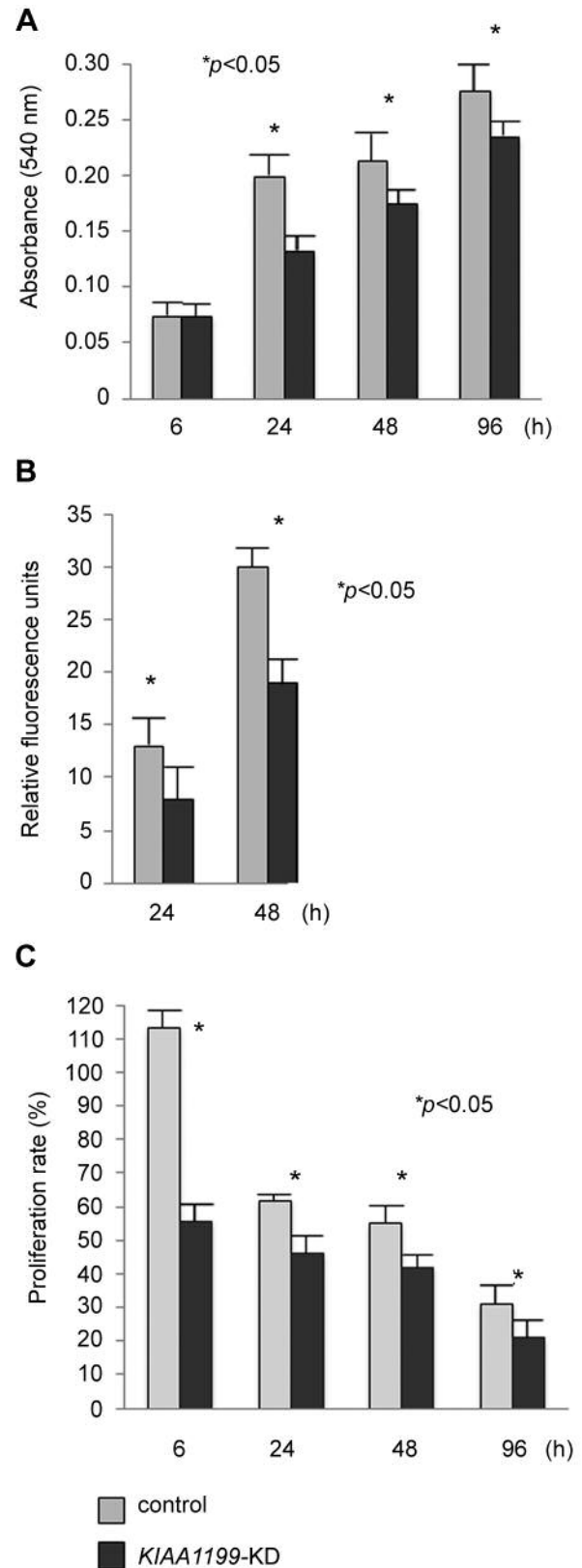


Figure 4. Changes in invasiveness (A), proliferation (B), and sensitivity to 5-FU (C) in KIAA1199-KD GC cells.

overexpression was correlated with lymph node metastasis, distant metastasis, and peritoneal dissemination in GC (15). However, we found no significant relationship between *KIAA1199* mRNA expression levels and any of the tested clinical features. In a previous report, *KIAA1199* mRNA overexpression was correlated with poor GC prognosis (15). Here, high *KIAA1199* mRNA expression was associated with poor outcomes in patients who underwent postoperative adjuvant chemotherapy with S-1 or adjuvant chemotherapy with S-1 alone, but not surgery alone. Furthermore, univariate-multivariate Cox proportional hazards model analysis showed that only high *KIAA1199* mRNA expression in cancer tissue was an independent poor prognostic factor in patients who underwent surgery and postoperative adjuvant chemotherapy with S-1. These results suggested that *KIAA1199* mRNA overexpression could lead to S-1 resistance. Indeed, we found that *KIAA1199*-KD in GC cells reduced their proliferative ability and invasiveness and increased sensitivity to 5-FU. To our knowledge, this is the first report to demonstrate that *KIAA1199* is related to 5-FU resistance in GC patients.

The relationship between *KIAA1199* mRNA expression and the poor outcomes in patients with locally advanced GC who underwent postoperative adjuvant chemotherapy with S-1 have yet to be fully elucidated. Our results along with previous studies suggest that four main factors are involved. First, *KIAA1199* expression is related to the proliferative ability of cancer cells *via* the Wnt/ $\beta$ -catenin pathway (12, 17). Second, it has been reported that *KIAA1199* promotes the release of calcium from the endoplasmic reticulum, which increases protein kinase  $C\alpha$  activity that plays an important role in cell migration and invasion (18). Third, *KIAA1199* promotes epithelial mesenchymal transition *via* epidermal growth factor receptor signal transmission (19). Epithelial mesenchymal transition is regulated by transforming growth factor- $\beta$ , which promotes cancer cell metastasis and drug-resistance (20). Finally, we found that *KIAA1199* may be associated with GC cell resistance to 5-FU. Taken together, these mechanisms suggest that high expression of *KIAA1199* mRNA in cancer tissue results in increased proliferation, invasion, cell migration, and chemotherapy resistance. This was associated with poor outcomes in patients with locally advanced GC, who underwent postoperative adjuvant chemotherapy with S-1.

Our study had several limitations. First, we only measured *KIAA1199* mRNA expression in GC tissue and we examined the relationship between mRNA expression in GC tissue and clinicopathological features or outcomes. Considering the clinical utility of *KIAA1199* as a biomarker, the relationship between its mRNA and protein levels should be examined using the same specimens. Second, GC tissues are heterogeneous and the mRNA was extracted from a GC tissue sample  $1\text{ cm}^2 \times 10\text{ }\mu\text{m} \times 3$  in size, including the deepest part, which means that this sample does not completely represent the characteristics of the entire tumor.

In conclusion, *KIAA1199* mRNA expression may be a useful prognostic marker in patients with locally advanced GC, who undergo postoperative adjuvant chemotherapy with S-1. *KIAA1199* may be a new target for GC pharmacotherapy because of its association with 5-FU resistance.

## Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

## Authors' Contributions

The study presented here was carried out in collaboration with all the Authors. Masataka Oneyama and Naoya Sakamoto contributed to the design, protocol creation, experiments, analysis of data, and writing of the manuscript. Naohide Oue contributed to the design. Yayoi Kimura contributed to the protocol creation and analysis of the data. Yukihiro Hiroshima contributed to the analysis of data. Itaru Hashimoto, Kentaro Hara, Yukio Maezawa, Kazuki Kano, Toru Aoyama, Hirohito Fujikawa, Takanobu Yamada, Hiroshi Tamagawa, Naoto Yamamoto, Takashi Ogata, Haruhiko Cho, Hiroyuki Ito, Norio Yukawa, Manabu Shiozawa, Takaki Yoshikawa, Soichiro Morinaga, and Yasushi Rino contributed to acquisition of samples. Munetaka Masuda contributed to writing of the manuscript. Yohei Miyagi contributed to the design, protocol creation, and writing of the manuscript. Wataru Yasui contributed to the design and writing of the manuscript. Takashi Oshima contributed to the design, protocol creation, analysis of data, acquisition of samples, and writing of the manuscript. All Authors read and approved the final manuscript.

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