Clinical significance of the *KRAS G13D* mutation in anastomotic recurrence of colorectal cancer

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Abstract. The genetic risk factors for anastomotic recurrence (AR) after curative surgery for colorectal cancer (CRC) are unclear. The present study is a single-center retrospective observational study that aimed to elucidate the association between the *KRAS G13D* mutation and AR in CRC. The present study included 21 patients with AR and 67 patients with non-anastomotic local recurrence (NALR) following curative surgery for CRC between January 2005 and December 2019. *KRAS G13D* mutation status was examined by droplet digital polymerase chain reaction. Data of clinicopathological findings and oncological outcomes were analyzed and compared between the AR group and the matched NALR group. The prevalence of the *KRAS G13D* mutation was significantly higher in the AR group (AR vs. NALR, 33.3 vs. 4.8%; P=0.047). Comparing the *KRAS G13D* mutation-positive and *KRAS G13D* mutation-negative patients in the AR group, there was no significant difference in the time from initial surgery to AR or resection rate of AR; however, all patients with *KRAS G13D* mutation who underwent resection of AR had subsequent recurrence within 2 years after resection, and overall survival was poor (3-year survival rate: Positive vs. negative, 68.6 vs. 90.9%; P=0.02). The prevalence of the *KRAS G13D* mutation was significantly higher in patients with AR, and *KRAS G13D*-mutant patients with AR had a poorer prognosis than those that were negative for the *KRAS G13D* mutation. In conclusion, postoperative surveillance and treatment strategies should be considered with attention to the possibility of AR and subsequent recurrence in *KRAS G13D*-mutant patients.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide in terms of the number of patients affected and the second most common in terms of the number of deaths, and its prevalence is increasing (1). Although a number of CRC cases can be cured by surgery, it is reported worldwide that recurrence occurs in ~30% of cases, even after curative resection (2), and prevention and early detection of recurrence are still major issues.

Local recurrence (LR) after resection for CRC is a serious issue. LR is defined as a recurrent lesion in or around the primary tumor site, including the pericolic tissue, the adjacent mesentery, lymph nodes or the suture line of anastomosis. Anastomotic recurrence (AR) has often been considered a type of LR, and its incidence has been reported to be 1-2% worldwide (3). AR has been shown to have a poorer prognosis and can progress to more advanced pathological stages than primary tumors (3). AR is thought to be caused by the implantation of exfoliated tumor cells into the anastomotic line, and the risk of AR is high in rectal cancer (3,4); however, to the best of our knowledge, the relationship with other clinicopathological factors has not been established. At present, there have been two reports on the genetic analysis of AR cases, but none of them have presented with any specific genetic features of AR (5,6). Our previous study reported on two cases of repeated AR following curative resection of CRC and both were revealed to have the *KRAS G13D* mutation (7). Although only two cases were reported, it may be hypothesized that the *KRAS G13D* mutation could contribute to the development of AR, as well as other aspects of recurrence, such as resistance and dormancy. The present study aimed to clarify the relationship between the *KRAS G13D* mutation and AR in CRC.

Patients and methods

Patients. The present study assessed 21 patients who underwent curative resection for CRC at the Department of Surgical Oncology, Faculty of Medicine, The University of Tokyo

(Tokyo, Japan) between January 2005 and December 2019, and were diagnosed with AR. A total of 67 patients who were diagnosed with non-anastomotic LR (NALR) after curative resection were also included. Patients with hereditary CRC and colitis-associated cancer were excluded from the study. In the present study, AR was defined as 'recurrence on the anastomotic line'; the recurrence site must be located on the anastomotic line and pathologically proven with a resection specimen or endoscopic biopsy. Recurrent lesions that were in contact with the anastomotic line, but mainly located outside of the bowel wall were not considered as AR and were classified as NALR. NALR did not include pelvic peritoneal dissemination or lateral lymph node recurrence in the present study. NALR was observed in 67 cases, and 21 cases of NALR matched to 21 cases of AR were used as a control group to compare the prevalence of the *KRAS G13D* mutation. In addition to the prevalence of the *KRAS G13D* mutation, the following clinicopathological findings were retrospectively evaluated: Sex, age, gross appearance type (classification of gross appearance), tumor size, histopathological type, tumor depth, lymph node metastasis, venous invasion, lymphatic invasion, preoperative carcinoembryonic antigen and carbohydrate antigen 19-9 levels and the association of the *KRAS G13D* mutation with prognosis (Table I). The clinicopathological findings were described according to the American Joint Committee on Cancer/International Union Against Cancer TNM classification, 8th edition (8).

The present study was conducted according to The Declaration of Helsinki and the study protocol was approved by the ethics committee of The University of Tokyo [approval no. 3252-(13)]. Informed consent was obtained in the form of an opt-out option on the website (http://all-1su.umin.jp/research/files/04_2.pdf).

DNA extraction from formalin-fixed, paraffin-embedded (FFPE) specimens. DNA was extracted from 10-μm FFPE specimens obtained from the University of Tokyo. A Maxwell® RSC DNA FFPE Kit (Promega Corporation) was used for DNA extraction. All samples were extracted after an overnight proteinase K digestion step at 70˚C, and all extractions were performed according to the manufacturer's protocol.

KRAS G13D mutation detection by droplet digital polymerase chain reaction (ddPCR). Mutation of the *KRAS* gene was examined by ddPCR using the QX200™ Droplet Digital™ PCR system (Bio-Rad Laboratories, Inc.). Each DNA sample was diluted to 3,000 ng/ml, as measured by a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Inc.). PCR reaction mixtures contained 12 μl ddPCR Supermix for Probes (Bio-Rad Laboratories, Inc.), 1.2 μl PrimePCR for ddPCR, 1.2 μl Uracil-DNA Glycosylase (UDG; New England BioLabs, Inc.) and 9.6 μl diluted DNA sample; 20 μl of the 24-μl reaction mixture was loaded in a DG8™ Cartridges for QX200™/QX100™ Droplet Generator (Bio-Rad Laboratories, Inc.) and droplets were generated. The entire droplet emulsion volume was further loaded in ddPCR™ 96-Well Plates (Bio-Rad Laboratories, Inc.). The loaded 96-well PCR plate was then heat-sealed with pierceable foil in the PX1™ PCR Plate Sealer and placed in a T100™ Thermal Cycler (both from Bio-Rad Laboratories, Inc.). Amplification was conducted as follows: 95˚C for 1 min, followed by 40 cycles at 55˚C for 10 min, 94˚C for 30 sec and a final extension step at 98˚C for 10 min. Commercial primers (PrimePCR for ddPCR *KRAS G13D*, assay ID: dHsaMDV2510598; Bio-Rad Laboratories, Inc.) were used. UDG was used to limit the chances of artifacts due to formalin-fixation (9).

Table I. Clinicopathological characteristics of patients with AR and NALR after matching.

Variable	AR (n=21)	NALR (n=21)	P-value
Sex			1.00
Male (%)	13 (61.9)	13 (61.9)	
Age			0.76
≥65 years (%)	10 (47.6)	11 (52.4)	
Tumor location			1.00
Rectum (%)	8 (38.1)	8 (38.1)	
Pre-operative CEA			0.53
≥5 ng/ml (%)	11 (52.4)	14 (66.7)	
Pre-operative CA 19-9			0.70
≥37 ng/ml (%)	3 (14.3)	5 (23.8)	
T stage			0.61
T3-4 (%)	20 (95.2)	18 (85.7)	
N stage			1.00
N1-2 (%)	15 (71.4)	15 (71.4)	
Tumor diameter			0.76
≥50 mm (%)	11 (52.4)	12 (57.1)	
Histological type			1.00
Tub (%)	20 (95.2)	20 (95.2)	
Lymphatic invasion			0.53
Positive (%)	10 (47.6)	8 (38.1)	
Venous invasion			0.41
Positive (%)	16 (76.2)	19 (90.5)	
Pathological stage			1.00
I-II (%)	6 (28.6)	6 (28.6)	
III (%)	11 (52.4)	12 (57.1)	
IV (%)	4 (19.0)	3 (14.3)	
KRAS G13D mutation			0.05
Positive (%)	7 (33.3)	1 (4.8)	

AR, anastomotic recurrence; NALR, non-anastomotic recurrence; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; Tub, tubular adenocarcinoma.

KRAS mutation detection in clinical practice. *RAS* mutation status (*KRAS* exon 2, 3, or 4 mutation, *NRAS* exon 2, 3, or 4 mutation) was evaluated using the PCR-reverse sequence-specific oligonucleotide method (BML, Inc.).

Statistical analysis. All statistical analyses were carried out using EZR version 4.1.2 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R version 3.0.2 (The R Foundation) (10). Comparisons were performed using χ^2 test or Fisher's exact test

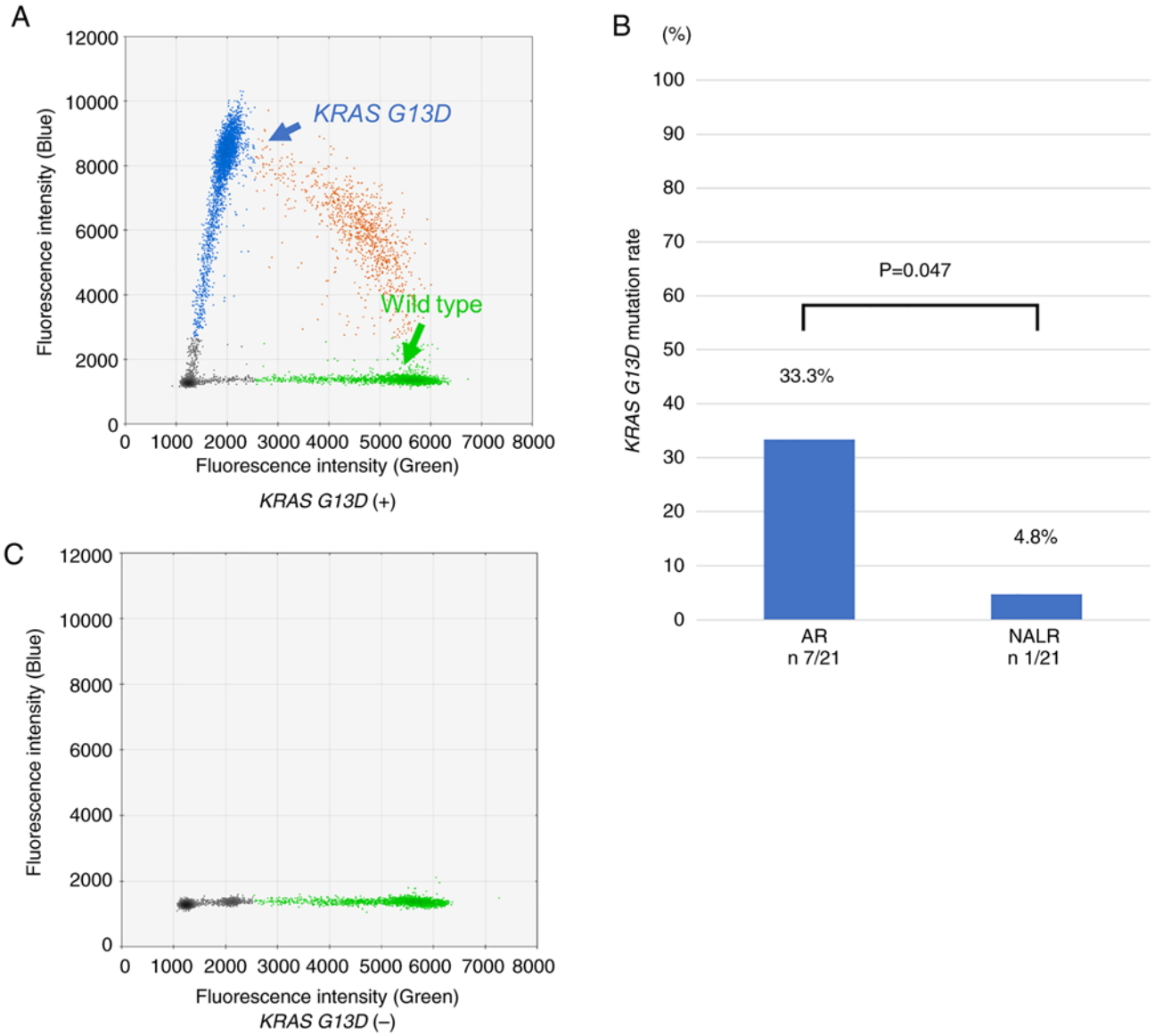

Figure 1. Detection of *KRAS G13D* mutation by droplet digital polymerase chain reaction. (A) *KRAS G13D* (+), Green cluster, wild-type *KRAS* droplets; blue cluster, *KRAS G13D* (+) droplets; orange cluster, droplets include both wild-type *KRAS* and *KRAS G13D* (+). (B) *KRAS G13D* mutation rate of patients with AR and NALR. (C) *KRAS G13D* (-). AR, anastomotic recurrence; NALR, non-anastomotic local recurrence; *KRAS G13D* (+), *KRAS G13D* mutation-positive; *KRAS G13D* (-), *KRAS G13D* mutation-negative.

for categorical variables. In univariate analysis using Fisher's exact test, odds ratio and their 95% confidence intervals were estimated. Survival curves were drawn using the Kaplan-Meier method and compared using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

To reduce potential confounding effects and treatment selection bias, case matching was conducted in the comparative analysis of AR and NALR. The following five factors that could affect *KRAS* status were selected: Age, sex, cancer location, histological type and cancer stage. R version 3.0.2 package 'optmatch (https://cran.r-project.org/web/packages/optmatch)' was used for the matching.

Results

The study population comprised 21 AR cases and 21 matched NALR cases. The clinicopathological characteristics of patients with AR and NALR before matching are shown in Table SI. The median follow-up period was 52.3 months. The clinicopathological characteristics of patients with AR or NALR after matching are shown in Table I.

There was no significant difference in the clinicopathological characteristics between the matched AR and the NALR groups, whereas the *KRAS G13D* mutation rate was significantly higher in the AR group; AR 33.3% (7/21) vs. NALR 4.8% (1/21) (P=0.047; Fig. 1).

The pathological findings on the recurrent lesions and details of treatment and prognosis in the AR group are shown in Table II. Of the 14 patients who tested negative for the *KRAS G13D* mutation in the present study, the *KRAS* status of 8 cases was evaluated for clinical purposes; 2 cases were positive for the *KRAS G12D* mutation, and the remaining 6 cases presented with wild-type *KRAS*. All of the 7 patients who tested positive for the *KRAS G13D* mutation were evaluated

Table II. Information on tumor recurrence and therapy for patients with AR.

No.	*KRAS G13D*	*RAS* status	Age, years	Sex	Tumor location	NAC	Surgery	Depth	Histology	pN	ly	v	AC	Anti-EGFR
1	+	*G13D*	78	F	Ascending	-	T/C	MP	Tub	1	2	2	-	-
2	+	*G13D*	61	M	Sigmoid	-	LAR	SE	Tub	1	0	3	+	-
3	+	*G13D*	33	F	Rectum	-	APR	SS	Tub	0	1	1	+	-
4	+	*G13D*	42	F	Sigmoid	-	LAR	SS	Tub	0	0	2	+	-
5	+	*G13D*	39	M	Rectum	+	LAR	MP	Tub	0	0	0	+	-
6	+	*G13D*	68	F	Ascending	-	-	-	Tub	-	-	-	-	-
7	+	*G13D*	59	M	Rectum	-	-	-	Por	-	-	-	-	-
8	-	*G12D*	66	M	Cecum	-	RHC	SE	Tub	0	0	0	-	-
9	-	*G12D*	63	M	Sigmoid	-	LAR	A	Tub	0	1	-	-	-
10	-	*WT*	55	F	Rectum	+	LAR	A	Tub	0	0	1	+	+
11	-	*WT*	77	M	Sigmoid	-	S/C	SI	Tub	1	1	0	+	-
12	-	*WT*	70	M	Rectum	-	LAR	A	Tub	1	0	1	+	+
13	-	*WT*	66	M	Descending	-	LHC	SS	Tub	1	2	2	-	-
14	-	*WT*	79	M	Sigmoid	+	LAR	A	Tub	0	0	1	-	-
15	-	*WT*	55	M	Rectum	-	-	-	Tub	-	-	-	-	-
16	-	Not tested	60	F	Rectum	-	Hartmann	A	Tub	0	0	0	+	-
17	-	Not tested	75	F	Sigmoid	-	S/C	MP	Tub	0	0	0	-	-
18	-	Not tested	60	M	Sigmoid	-	LHC	SS	Tub	0	1	2	+	-
19	-	Not tested	67	M	Sigmoid	+	APR	A	Tub	0	0	1	+	-
20	-	Not tested	69	F	Transverse	-	RHC	SE	Tub	1	0	3	-	-
21	-	Not tested	60	M	Rectum	-	-	-	Tub	-	-	-	-	-

AR, anastomotic recurrence; G13D, *KRAS G13D* mutant; G12D, *KRAS G12D* mutant; WT, *KRAS* wild-type; NAC, neoadjuvant chemotherapy before resection for AR; T/C, transverse colectomy; LAR, low anterior resection; APR, abdominoperineal resection; RHC, right hemicolectomy; S/C, sigmoid colectomy; LHC, left hemicolectomy; MP, muscularis propria; SE, serosa; SS, subserosa; A, the rectal cancer has grown into the outermost layers but has not gone through them; SI, the colon cancer has grown into other nearby tissues or organs; pN, pathological N stage; ly, lymph vascular invasion; v, venous invasion; AC, adjuvant chemotherapy after resection for AR; anti-EGFR, anti-epidermal growth factor receptor antibody; Tub tubular adenocarcinoma, Por; poorly differentiated adenocarcinoma; F, female; M, male; +, Yes; -, No.

Table III. Clinicopathological characteristics of patients with AR.

Variable	*KRAS G13D* (+) (n=7)	*KRAS G13D* (-) (n=14)	P-value
Sex			0.346
Male (%)	3 (42.9)	10 (71.4)	
Age			0.183
≥65 years (%)	2 (28.6)	9 (64.3)	
Tumor location			1.000
Rectum (%)	3 (42.9)	5 (35.7)	
Pre-operative CEA			0.362
≥5 ng/ml (%)	5 (71.4)	6 (42.9)	
Pre-operative CA 19-9			0.527
≥37 ng/ml (%)	2 (28.6)	1 (7.14)	
T stage			0.333
T3-4	7 (100)	13 (92.9)	
N stage			0.613
N1-2	6 (85.7)	9 (64.3)	
Tumor diameter			0.362
≥50 mm (%)	5 (71.4)	6 (42.9)	
Histological type			0.333
Tub (%)	6 (85.7)	14 (100)	
Lymphatic invasion			1.000
Positive (%)	3 (42.9)	7 (50.0)	
Venous invasion			1.000
Positive	5 (71.4)	11 (78.6)	
Pathological stage			0.589
I-II	1	5	
III	4	7	
IV	2	2	
Simultaneous other metastases			1.000
Yes	3 (42.9)	6 (42.9)	
Resection for AR			0.574
Yes	5 (71.4)	12 (85.7)	
Use of anti-EGFR antibody			0.533
Yes	0 (0.0)	2 (14.3)	

AR, anastomotic recurrence; EGFR, epidermal growth factor; *KRAS G13D* (+), *KRAS G13D* mutation-positive; *KRAS G13D* (-), *KRAS G13D* mutation-negative; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; Tub, tubular adenocarcinoma.

for *KRAS* status in the clinical setting, and there were no cases with double *KRAS* mutations. The *RAS* status of the matched NALR group is shown in Table SII. Of the 20 patients who tested negative for the *KRAS G13D* mutation, the *KRAS* status of 10 cases was evaluated for clinical purposes; three cases were positive for the *KRAS G12D* mutation, two cases were positive for the *KRAS G12V* mutation, one case was positive for the KRAS G12A mutation and the remaining four cases presented with wild-type *KRAS*.

Comparing the *KRAS G13D* mutation-positive (*KRAS G13D*$^+$) and *KRAS G13D* mutation-negative (*KRAS G13D*$^-$) patients in the AR group, there was no significant difference in the clinicopathological background (Table III), interval from initial surgery to AR (Fig. 2A) and recurrence resection rate (Table III). On analyzing the 17 cases who underwent surgical resection of AR, even though there was no significant difference in recurrence-free survival (RFS) after resection (2-year RFS after resection: *KRAS G13D*$^+$ 0% vs. *KRAS G13D*$^-$ 33.3%; P=0.10), all *KRAS G13D*$^+$ patients experienced subsequent recurrence within 2 years (Fig. 2B). Notably, *KRAS G13D*$^+$ patients had a significantly poorer overall survival (OS) (3-year OS: *KRAS G13D*$^+$ 68.6% vs. *KRAS G13D*$^-$ 90.9%; P=0.02) (Fig. 2C). The rate of synchronous recurrence at the diagnosis of AR was 28.6% (2/7) in *KRAS G13D*$^+$ patients and 42.9% (6/14) in *KRAS G13D*$^-$ patients. There was no significant difference in the synchronous recurrence patterns between patients with and without the *KRAS G13D* mutation (Table IV). By contrast,

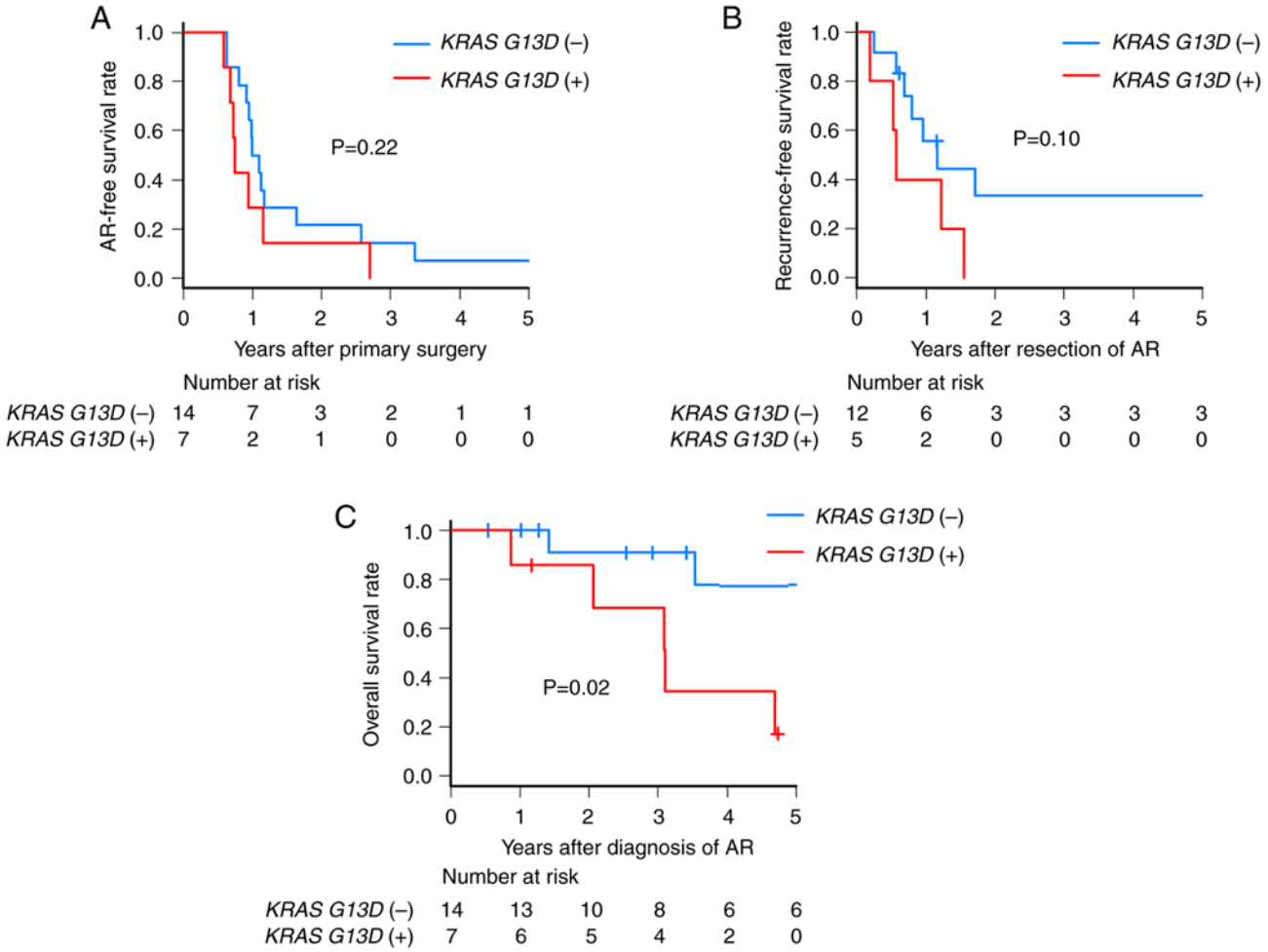

Figure 2. Kaplan-Meier curves of interval to AR, recurrence-free and overall survival according to *KRAS G13D* status. (A) Interval from the initial surgery to AR. (B) Recurrence-free survival from resection for AR. (C) Overall survival from diagnosis of AR. AR, anastomotic recurrence; *KRAS G13D* (+), *KRAS G13D* mutation-positive; *KRAS G13D* (-), *KRAS G13D* mutation-negative.

KRAS G13D^{+} patients tended to experience more instances of metachronous locoregional recurrences (P=0.10), and repetitive AR was observed in *KRAS G13D*$^{+}$ patients alone (P=0.07) (Table V); 80.0% (4/5) of the *KRAS G13D*$^{+}$ patients experienced subsequent distant metastatic recurrences.

Discussion

The present study observed a high *KRAS G13D* mutation rate in patients with AR (AR 33.3% vs. matched NALR 4.8%; P=0.047). All *KRAS G13D*$^{+}$ patients who underwent curative resection for AR had subsequent recurrence within 2 years, and the OS was poorer than that of *KRAS G13D*$^{-}$ patients (3-year OS: 68.6% vs. 90.9%; P=0.02).

KRAS is one of the key driver genes in CRC and is detected early in the carcinogenesis of CRC. The *KRAS* mutation rate in CRC is reported to be 30-40% worldwide, and *KRAS* codon 13 mutations, including *KRAS G13D*, are reported to occur in 6-8% of cases worldwide (11-13). In a large-scale study in Japan, the total *KRAS* mutation rate was 37.6%, and the *KRAS* codon 13 mutation rate was 7.7%, which was comparable to the results reported in Western countries (14).

The 33.3% *KRAS G13D* mutation rate among patients with AR in the present study was higher than that reported in previous studies, suggesting that there may be a certain association between the *KRAS G13D* mutation and AR. In addition, there was a significant difference in the *KRAS G13D* mutation rate between AR and NALR, suggesting that this is a specific characteristic of AR rather than of LR. Andreyev *et al* (11) reported that G to A mutations in the *KRAS* gene, which include the *KRAS G13D* mutation, were more frequent (58.3%) among patients with AR than among patients with other types of recurrence (~22%) (P=0.02), and the results of this previous study are consistent with the current findings.

Although several reports have stated that CRC with *KRAS* mutations has a poor prognosis (14-16), there are few reports on the association between prognosis and each *KRAS* subtype, and consistent results have not been obtained (17-20). Notably, the association between *KRAS* subtypes and the clinical significance of CRC has been reported in few studies. Kodaz *et al* (21) reported that the *KRAS G13D* mutation was more frequent in the left colon and in patients <70 years old, whereas the *KRAS G12D* mutation was more frequent in the right colon and in patients >50 years old. Bazan *et al* (18) reported that codon 12 *KRAS* mutations were associated with mucinous histology, whereas codon 13 *KRAS* mutations were associated with lymph node metastasis and advanced Dukes' stage.

Table IV. Distribution of synchronous recurrent sites of patients with AR.

Recurrent sites	*KRAS G13D* (+) (n=7)	*KRAS G13D* (-) (n=14)	OR (95% CI)	P-value
Locoregional (%)	0 (0.0)	0 (0.0)	-	-
Distant metastasis (%)	2 (28.6)	6 (42.9)	0.549 (0.039-5.020)	0.66
Liver	1 (14.3)	5 (35.7)	0.316 (0.005-4.004)	0.61
Lung	1 (14.3)	1 (7.1)	2.082 (0.023-182.6)	1.00
Dissemination	1 (14.3)	2 (14.3)	1.000 (0.015-23.10)	1.00
Extra-regional LN	0 (0.0)	0 (0.0)	-	-
Total (%)	2 (28.6)	6 (42.9)	0.549 (0.039-5.020)	0.66

AR, anastomotic recurrence; *KRAS G13D* (+), *KRAS G13D* mutation-positive; *KRAS G13D* (-), *KRAS G13D* mutation-negative; OR, odds ratio; CI, confidence interval; LN, lymph node. OR and 95% CI were estimated using Fisher's exact test.

Table V. Distribution of subsequent recurrent sites in patients with AR.

Recurrent sites	*KRAS G13D* (+) (n=5)	*KRAS G13D* (-) (n=12)	OR (95% CI)	P-value
Locoregional (%)	4 (80.0)	3 (25.0)	10.05 (0.668-651.0)	0.10
Anastomotic (repetitive)	2 (40.0)	0 (0.0)	Inf (0.495-Inf)	0.07
Non-anastomotic	2 (40.0)	3 (25.0)	1.915 (0.110-28.29)	0.60
Distant metastasis (%)	4 (80.0)	5 (41.7)	5.059 (0.352-313.6)	0.29
Liver	0 (0.0)	2 (16.7)	0 (0-13.32)	1.00
Lung	2 (40.0)	1 (8.3)	6.321 (0.251-468.8)	0.19
Dissemination	1 (20.0)	2 (16.7)	1.233 (0.017-30.77)	1.00
Extra-regional LN	1 (20.0)	1 (8.3)	2.569 (0.028-234.6)	0.52
Total (%)	5 (100.0)	7 (58.3)	Inf (0.410-Inf)	0.25

AR, anastomotic recurrence; *KRAS G13D* (+), *KRAS G13D* mutation-positive; *KRAS G13D* (-), *KRAS G13D* mutation-negative; OR, odds ratio; CI, confidence interval; LN, lymph node; Inf, infinity.

In contrast to previous reports, the present study focused on AR cases, which may be responsible for the difference in prognosis between the cases with *KRAS G13D* mutation and those without the mutation. Several *in vitro* analyses have shown the characteristics of *KRAS* subtypes using colon cancer cell lines. Organ *et al* (22) reported that DLD1, a colon cancer cell line that is positive for the *KRAS G13D* mutation, showed a higher adhesion ability to the extracellular matrix and migration, in contrast to DKO4, a cell line in which *KRAS G13D* was knocked out of DLD1 cells. It was hypothesized that the *KRAS G13D* mutant may have an enhanced adhesion ability to the extracellular matrix and migration compared with wild-type *KRAS*, and these characteristics may contribute to the implantation of tumor cells into the anastomotic line, which is the pathogenic mechanism of AR. Stolze *et al* (23) reported that *KRAS G13D*, unlike other subtypes of *KRAS* mutation, showed a high expression of epidermal growth factor receptors (EGFRs) and high activation of proliferative signaling in the presence of EGF. In the tissue repair process at the anastomotic site, the role of growth factors is important; therefore, these characteristics of the *KRAS G13D* mutation may be responsible for AR.

In the present study, *KRAS G13D*$^+$ patients with AR had a poor prognosis, probably because all *KRAS G13D*$^+$ patients with AR experienced a subsequent recurrence after undergoing resection for AR. Margonis *et al* (24) reported that the *KRAS* codon 13 mutation was a risk factor for extrahepatic and pulmonary recurrence after curative resection of liver metastasis of CRC, and this was not observed for all *KRAS* mutations. Owing to the small number of AR cases in the present study, statistically significant difference was not be observed; however, it was observed that 80% (4/5) of the *KRAS G13D*$^+$ patients experienced subsequent distant metastatic recurrence after curative resection for AR. As aforementioned, it has been reported that the *KRAS G13D* mutation enhances the adhesion and migratory ability, and is associated with increased proliferative signaling. These characteristics may be responsible for the pathogenesis of AR, in addition to the subsequent recurrences and poor prognoses.

The poor prognosis of *KRAS G13D*$^+$ patients could also be attributed to the absence of an indication for a regimen including anti-EGFR antibodies (21). However, only 2 of the 14 patients with AR diagnosed as *KRAS G13D*$^-$ received chemotherapy including anti-EGFR antibodies in the present study; therefore, the effect of anti-EGFR antibodies may be limited. Few reports have suggested that the *KRAS G13D* mutation differs from other *KRAS* subtypes and may benefit from

treatment with the anti-EGFR antibody cetuximab (25,26), but this has been doubted by some reports (27) and no conclusion has been reached. The therapeutic efficacy of anti-EGFR antibody therapy in *KRAS G13D*$^{+}$ requires further study.

There were several limitations to the present study. First, it was a single-center, retrospective study with a small number of patients. Second, the study only analyzed the *KRAS G13D* mutation and did not consider other *KRAS* subtypes or *BRAF* mutations. Although 8 *KRAS G13D*$^{-}$ patients were tested for the *RAS* status in the clinical setting, 6 *KRAS G13D*$^{-}$ patients were not tested. Therefore, the *KRAS G13D*$^{-}$ group may have included patients with other *KRAS* subtypes or *BRAF* mutations. Additionally, 2 cases belonging to the *KRAS G13D*$^{-}$ group possessed the *KRAS G12D* mutation. Third, there was no analysis of the effect of AR on patient survival compared with the patients with NALR.

In conclusion, the *KRAS G13D* mutation rate was significantly higher in patients with AR, and patients with AR and the *KRAS G13D* mutation had a poorer prognosis than *KRAS G13D*$^{-}$ patients with AR. Although the role of the *KRAS G13D* mutation in the development of AR requires further investigation, postoperative surveillance and treatment strategies should be considered with attention to the possibility of AR and subsequent recurrence in *KRAS G13D* mutation-positive patients. Although a definitive conclusion could not be reached due to the small sample size and the fact that it was not considered that mutations other than *KRAS G13D* may affect the outcome, the present results may be worth confirming in future studies containing a larger number of patients.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to a license agreement with the University of Tokyo, but are available from the corresponding author on reasonable request.

Authors' contributions

KeM, KS and KH substantially contributed to the study conception, design and analysis of data. KeM, KU, SK and TY substantially contributed to the acquisition of laboratory data. HN, KK, KoM, SE, YY, HS substantially contributed to the acquisition of clinical data. HY and SI substantially contributed to the interpretation of data. KeM and KS confirm the authenticity of all the raw data. HY and SI gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of The University of Tokyo [approval no. 3252-(13); Tokyo, Japan]. This study was conducted in accordance with The Declaration of Helsinki.

Patient consent for publication

Informed consent was obtained in the form of an opt-out option on the website for the participation in the research (http://all-1su.umin.jp/custom8.html).

Competing interests

The authors declare that they have no competing interests.

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