Association between KRAS G13D mutations and anastomotic recurrence in colorectal cancer
Two case reports

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Abstract

Rationale: The prevalence of anastomotic recurrence (AR) in colorectal cancer (CRC) after resection of the primary tumor (PT) is 5% to 14%. However, no association has been observed between specific somatic genetic alterations and AR. Such associations may shed light on the mechanism of AR.

Patient concerns: We experienced 2 patients with AR of CRC. The first patient was a 42-year-old woman who underwent resection of an AR of rectal cancer 2 times within 19 months after resection of the PT. The second patient was a 77-year-old woman who underwent resection of an AR of ascending colon cancer twice within 38 months after resection of the PT.

Diagnosis: Both cases were diagnosed as repetitive AR.

Interventions: Loss of heterozygosity analysis, microsatellite instability (MSI) study of 9 microsatellite loci, and mutational analysis of KRAS, BRAF, APC, TP53, and SMAD4 were performed.

Outcomes: All the lesions, except 1, harbored mutations in APC, KRAS, and TP53, without MSI, after neoadjuvant chemoradiotherapy. The APC, KRAS, and TP53 mutations were pathogenic or likely pathogenic in the PTs and ARs. Both women harbored the same KRAS G13D mutation, which accounts for 8% of all KRAS mutations in sporadic CRCs. The probability of the incidental occurrence of KRAS G13D mutations in both cases is 0.64%, provided that the mutations were independent of AR.

Lessons: Our findings may shed light on the mechanism of AR in CRC, namely, that the PT harbored the same mutations as the AR and the lesions in both cases harbored the KRAS G13D mutation.

Abbreviations: AR = anastomotic recurrence, CIN = chromosomal instability, CRC = colorectal cancer, CRT = chemoradiotherapy, CT = computed tomography, DST = double-stapling technique, LOH = loss of heterozygosity, MSI = microsatellite instability, MSS = microsatellite stable, PCR = polymerase chain reaction, PT = primary tumor, WT = wild type.

Keywords: anastomotic recurrence, colorectal neoplasms, genetic alterations, KRAS G13D

1. Introduction

Anastomotic recurrence (AR) is a form of local recurrence in colorectal cancer (CRC). The incidence of AR is reportedly 5% to 14%.[1–3] Despite the performance of rectal lavage and the acquisition of sufficient surgical margin, as well as other preventive measures.

There are 2 major hypotheses regarding the pathogenesis of AR. One pertains to the implantation of cancer cells in the suture line, and the other to the metachronous development of another primary tumor (PT) in an area of proliferative instability.[4,5] However, the precise mechanisms and causes of AR remain unclear. Another possible hypothesis is that cancer cells are biologically more aggressive and adhesive to the bare area of the anastomotic site in the case of AR. To the best of our knowledge, only 2 reports to date have investigated the genomic profile of PTs and ARs.[6,7] Costi et al investigated the microsatellite instability (MSI) and loss of heterozygosity (LOH) at 7 sites (Myc-L, BAT26, BAT40, DSSS346, D18S452, D18S64, and D16S402) in the PTs and ARs of 18 patients.[6] They observed LOH at 5q21 and/or 18p11.23 in both the PT and AR in more than half of the cases; this result was similar to that observed in the control group. Vakini et al used next-generation sequencing to determine the mutational status of cancer-associated genes. They reported that the PT and AR shared between 50% and 100% of the mutations in 13 microsatellite stable (MSS) lesions.[7] No specific somatic genetic alterations have been identified in association with AR to date. Herein, we report 2 cases of repetitive AR with somatic genetic analyses.

Editor: N.A.

This research is supported by Grants-in-Aid for Scientific Research (C: grant number 16K07143, C: grant number 16K07161, C: grant number 17K10620, C: grant number 17K10621, C: grant number 17K10623, and C: grant number 18K07194) from the Japan Society for the Promotion of Science. This research is supported by the Project for Cancer Research and Therapeutic Evolution (P-CREATE, grant number 18cm0106502h0003) from the Japan Agency for Medical Research and Development (AMED).

The authors declare that they have no conflicts of interest.

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Medicine (2019) 98(12):e14781

http://dx.doi.org/10.1097/MD.0000000000014781
2. Case presentation

2.1. Case 1

A 42-year-old woman visited the hospital with complaints of rectal bleeding, nausea, and left abdominal pain. Colonoscopy revealed a full circumferential cancerous lesion (1-PT) in the recto-sigmoid colon. High anterior resection using the double-stapling technique (DST) was performed. Pathological examinations showed a moderately differentiated adenocarcinoma (T3, N1b, M0) according to the Union for International Cancer Control tumor-node-metastasis classification. After surgery, the patient received 8 cycles of capecitabine plus oxaliplatin adjuvant therapy. Eight months after the initial surgery, the patient experienced rectal bleeding. Colonoscopy revealed AR (1-AR1) of a semi-circumferential tumor at the anastomotic site. Local resection of the AR was performed using the DST. The pathological examination showed a moderately differentiated adenocarcinoma (T3, N0, M0), which was compatible with AR. Nine months after the second surgery, which corresponded to 17 months after the initial surgery, the patient again experienced rectal bleeding, and colonoscopy revealed another recurrence of a circumferential tumor (1-AR2) at the anastomotic site. Preoperative chemoradiotherapy (CRT) was performed along with paraaortic lymph node dissection. Pathological examination showed that the recurrent lesions were both moderately differentiated adenocarcinomas (T2, N1a, M0). After the surgery, the patient received a regimen of tegafur–uracil as adjuvant therapy. 

2.2. Case 2

A 77-year-old woman with ascending colon cancer (2-PT) underwent right hemicolectomy with a functional end-to-end anastomosis at another hospital. Pathological examination revealed a moderately differentiated adenocarcinoma (T3, N1b, M0). Three years after the initial surgery, colonoscopy revealed tumor recurrence at the anastomotic site. It was diagnosed as AR (2-AR1), and surgery was performed. During the surgery, paraaortic lymph node metastasis was suspected, and local resection of the anastomotic site with side-to-end anastomosis was performed along with paraaortic lymph node dissection. Pathological examination showed a moderately differentiated adenocarcinoma (T3, N2b, M1a [paraaortic lymph node]). The histopathology of the recurring lesions resembled that of the PT.

Four years after the initial surgery (1 year after the second surgery), colonoscopy revealed a recurrent lesion at the anastomotic site and another recurrence at the stump of the side-to-end anastomosis. The patient received a CT scan, which showed no evidence of distant metastases. A second AR (2-AR2) was diagnosed. Local resection of the anastomotic site with side-to-end anastomosis was performed. Pathological examinations showed that the recurrent lesions were both moderately differentiated adenocarcinomas (T2, N1a, M0). After the surgery, the patient received a regimen of tegafur–uracil as adjuvant therapy.

Five months after the third surgery, lung metastases were detected by a whole-body CT scan. Resection of the lung metastases was performed, and postoperative follow-up was performed on an outpatient basis. The lung metastasis recurred 1 year and 8 months after resection. Because the patient was old and we did not want to administer FOLFOX, FOLFIRI, or molecularly targeted agents due to the increased risk of toxicity, tegafur–uracil was administered. However, the patient died due to disease progression 3 years and 8 months after the third operation.

2.3. DNA and RNA analysis

Tumor tissues and corresponding normal mucosae were obtained from the surgically resected specimens, and were either snap frozen or immersed in RNA protect tubes (Qiagen, Valencia, CA) overnight at 4°C, followed by storage at −20°C until use. Genomic DNA was extracted from the frozen tissue samples using the QiAamp DNA mini kit (Qiagen) according to the manufacturer’s instructions, or from formalin-fixed, paraffin-embedded tumor samples using the TaKaRa DEXPAT® Easy kit (TaKara Bio, Shiga, Japan) according to the manufacturer’s instructions. Total RNA was extracted using Trizol reagent (Invitrogen, ThermoFisher Scientific, Carlsbad, CA), and treated with DNase I (TaKara Bio) according to the manufacturer’s instructions.

2.4. Microsatellite instability and LOH analysis

Using the extracted DNA, we analyzed the MSI and LOH status through polymerase chain reaction (PCR) using AmpliTaq Gold DNA polymerase (Applied Biosystems, ThermoFisher Scientific, Foster City, CA) at 9 microsatellite loci: BAT25, BAT26, D2S123, DSS346, D17S250, TP53, D18S546, D18S563, and D18S474. We previously reported that 18q is a prognostic marker for CRC.[8] We defined LOH at each locus as a 50% reduction in the height of one of the 2 allele peaks in the tumor DNA relative to the non-neoplastic control DNA. We also investigated the LOH ratio, which is known to predict the worst survival for CRC.[9]

We defined the LOH ratio (%) as

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\text{LOH ratio(%) = } \frac{\text{Total number of chromosomes with LOH}}{\text{Total number of chromosomes that could be evaluated for LOH}} \times 100
\]

to evaluate the severity of chromosomal instability (CIN) based on the actual frequency of LOH among the evaluable loci. A LOH ratio ≥33% were classified as CIN high and an LOH ratio <33% was classified as CIN low.[9]

2.5. Mutation analysis

We examined the mutational status using Sanger sequencing. Using extracted DNA or complementary DNA prepared from the DNase I-treated RNA and PrimeScript RT master mix (TaKara Bio), we performed PCR using PrimeSTAR GXL DNA polymerase (TaKara Bio) according to the manufacturer’s instructions. We designed primers for KRAS (codons 12, 13, 59, 61, 117, and 146), BRAF (codon 600), APC (codons 1282–1381), TP53 (codons 1-345), and SMAD4 (codons 1-170 and 350-553) using Primer3Plus (https://primer3plus.com/). The amplified PCR products were cleaned up using ExoSAP-IT® (Applied Biosystems), cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and analysis was conducted using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). If any mutation was present, we analyzed whether the mutation was pathogenic using the Catalogue of Somatic Mutations in Cancer (COSMIC) (Sanger Institute, UK) or ClinVar (NCBI) databases.
2.6. Clinicopathological features of the PTs and ARs

The clinicopathological features of the PTs and ARs are shown in Table 1. The histopathological diagnoses of the PT and recurrent specimens were concordant (moderately differentiated adenocarcinoma) in both Case 1 and Case 2. Both cases were positive for lymph node metastasis at the time of PT resection. The colonoscopy findings and clinical course of each of the cases are summarized in Figure 1.

2.7. Microsatellite instability and LOH analysis

The results of the analysis for the 9 microsatellite markers are shown in Table 2. Both patients showed an MSS status in the PT and ARs. In Case 1, we observed LOH at the 5q and 18q loci in the PT (1-PT) and the first AR (1-AR1). However, it was not observed in the second AR (1-AR2). In Case 2, LOH was observed only at 17p in the first AR (2-AR1). PT specimens were not available. LOH was not observed in the second AR (2-AR2).

We also evaluated the LOH ratio for each tumor. In Case 1, the LOH ratio of the PT (1-PT) and first AR (1-AR1) was 50% (CIN high). However, that of the second AR (1-AR2) was 0% (CIN low). In Case 2, the LOH ratios of the first AR (2-AR1) and second AR (2-AR2) were both 0% (CIN low).

2.8. Mutation analysis

The results of sequence analyses of KRAS, BRAF, APC, TP53, and SMAD4 are shown in Table 3.

In Case 1, the PT (1-PT) and first AR (1-AR1) shared KRAS (p. G13D), APC (p.E1353*), and p53 (p.V272L) mutations. The BRAF and SMAD4 mutations in the indicated codons were wild type (WT) in both the tumors. However, there were no mutations in the second AR (1-AR2). The KRAS (p.G13D) and APC (p. E1353*) mutations were pathogenic, according to COSMIC. The p53 (p.V272L) mutation was also likely pathogenic.

In Case 2, the first AR (2-AR1) and second AR (2-AR2) shared the KRAS (p.G13D) mutation. In the second AR (2-AR2), both the tumors shared the APC (p.L1488fs) and p53 (p.P152L) mutations. In both the tumors, the BRAF and SMAD4 genotypes in the indicated codons were WT. The KRAS (p.G13D), APC (p. L1488fs), and p53 (p.P152L) mutations were pathogenic, according to COSMIC. The PT (2-PT) specimens were not available.

2.9. Ethical review

This study was approved by the Ethics Committee of the University of Tokyo (No. G3552-13).

Informed written consent was obtained from the patients for publication of this case report and accompanying images.

3. Discussion and conclusions

We reported 2 cases of repetitive CRC AR with genetic analyses. In both cases, all the lesions except for 1, which was discovered after neoadjuvant CRT, harbored APC, KRAS, and TP53 mutations without MSI; this probably corresponds to the consensus molecular subtype.[10] Interestingly, the tumors shared the same KRAS G13D mutation, which accounts for 8% of all KRAS mutations in sporadic CRC. The probability of the incidental occurrence of KRAS G13D mutations in both cases is 0.64%, provided that KRAS G13D mutations are independent of AR. The relationship between KRAS mutational patterns and AR development has not been reported before. Few studies have investigated the genetic alterations associated with AR in CRC,[6,7] and our findings may shed light on the mechanism of AR in such cases.

All the lesions except the lesion resected after adjuvant CRT (1-AR2) showed a good chemoradiation effect, and the sample may have contained fewer cancer cells and a higher number of normal cells, resulting in the negative results of the genetic analysis. This finding was in line with a previous report by Vakini et al,[7] who performed next-generation sequencing of 36 tumors from 14 patients and defined the mutational status of 341 cancer-associated genes. They demonstrated that the lesions displayed an

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**Table 1**

Clinicopathological features of the primary tumors and anastomotic recurrences.

| Case/Age (yr)/Sex | Number of recurrences | Tumor location | Anastomotic technique | Interval after initial surgery | Treatment | Histological diagnosis of the primary tumor and recurrent specimen |
|-------------------|-----------------------|----------------|------------------------|------------------------------|-----------|---------------------------------------------------------------|
| 1/42/F            | 1                     | Rectosigmoid colon | DST                    | 8 months                     | High anterior resection | Moderately differentiated adenocarcinoma–T3, N1b, M0 |
|                   |                       |                 |                        |                              |            | Moderately differentiated adenocarcinoma–T3, N0, M0          |
|                   | 2                     | Anastomosis     | DST                    | 1 year, 6 months            | Resection of the anastomosis | Moderately differentiated adenocarcinoma–T4b (uterus invasion–N0, M0), Circumferential resection margin positive |
|                   |                       |                 |                        |                              | Hartmann’s operation after neoadjuvant chemoradiotherapy | Moderately differentiated adenocarcinoma–T3, N1b, M0 |
|                   |                       |                 |                        |                              | Re-resection of the anastomosis | Moderately differentiated adenocarcinoma–T3, N2b, M1a (paraaortic lymph node) |
| 277/F             | 1                     | Ascending colon | FEEA                   | 3 years                      | Resection of the anastomosis | Moderately differentiated adenocarcinoma–T2, N1a, M0 |
|                   | 2                     | Anastomosis     | Side-to-end anastomosis | 4 years                      | Resection of the anastomosis | Moderately differentiated adenocarcinoma–T2, N1a, M0 |

DST = double-stapling technique, FEEA = functional end-to-end anastomosis.
MSS pattern in 13 patients and had a higher KRAS mutation rate (69%), without reaching significance. Unfortunately, the authors did not present detailed information on KRAS mutational patterns, such as codon positions and amino acid substitution patterns. Costi et al performed MSI and LOH analyses of 7 sites (Myc-L, BAT26, BAT40, D5S346, D18S452, D18S64, and D16S402) in the PTs and ARs of 18 patients, and demonstrated that more than half of the cases had lesions with CIN.[6] The mutation pattern of KRAS was concordant in terms of amino acid substitution (G13D) in both cases in the present study. The reported incidence of KRAS mutation is approximately 40% among all colorectal tumors.[11] KRAS mutations frequently occur at codon 12 (G12D, 13%; G12V, 9%) or 13 (G13D, 8%). The probability of the incidental occurrence of KRAS G13D mutations in both cases is 0.64%, which is too rare for it to be independent of AR. Therefore, we hypothesized that

![Table 2](image)

Table 2
Molecular alterations in the primary colon cancer and anastomotic recurrences.

| Case | Sample  | Tumor location       | Template          | MSS  | c-kit | hMSH2 | hMSH2,6 | APC  | BRCA1 | TP53 | DCC  | SMAD4  | SMAD4  | 2p  | 2p  | 5q  | 17p | 17p | 18q | 18q | 18q |
|------|---------|----------------------|-------------------|------|-------|-------|---------|------|-------|------|------|--------|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1    | PT      | Rectosigmoid colon   | Frozen/gDNA       | MSS  | dS2   | dS2   | D2S123  | DSS346 | D17S250 | TP53 | D18S46 | D18S46 | D18S46 | 5q  | 5q  | 17p | 17p | 18q | 18q | 18q | 18q |
|      | AR1     | Anastomosis          | Frozen/gDNA       | MSS  | dS2   | RET   | LOH     | RET   | N.I.   | LOH  | LOH  | LOH    | LOH    | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. |
|      | AR2     | Anastomosis          | Frozen/gDNA       | MSS  | RET   | RET   | RET     | N.I.   | RET   | RET   | RET   | RET    | RET    | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. |
| 2    | PT      | Ascending colon      | FFPE/fixed, paraf  | MSS  | N.I.  | N.I.  | N.I.    | N.I.   | N.I.   | RET   | N.D.  | N.I.   | N.D.   | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. |
|      | AR1     | Anastomosis          | FFPE/fixed, paraf  | MSS  | N.I.  | RET   | RET     | N.D.   | RET   | RET   | RET   | RET    | RET    | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. |
|      | AR2     | Stump                | Frozen/gDNA       | MSS  | N.I.  | RET   | RET     | N.I.   | RET   | RET   | RET   | RET    | RET    | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. | N.I. |

AR = anastomotic recurrence, FFPE = formalin-fixed, paraffin-embedded, gDNA = genomic DNA, LOH = loss of heterozygosity, MSS = microsatellite stable, N.D. = not determined, N.I. = not informative, PT = primary tumor, RET = retention of heterozygosity.
Table 3
Mutations in the primary tumor and anastomotic recurrences.

| Case | Sample   | Tumor location | Template          | KRAS Codons 12, 13, 59, 61, 117, 146 | BRAF Codon 600 | APC Codons 1282–1581 | TPS3 Codons 1–345 | SMAD4 Codons 1–170, 350–553 |
|------|----------|----------------|-------------------|--------------------------------------|----------------|----------------------|--------------------|--------------------------|
| 1    | PT       | Rectosigmoid colon | Frozen/gDNA     | –                                    | –              | –                    | –                  | –                       |
| AR1  | Anastomosis | Frozen/gDNA | cDNA G13D      | –                                    | –              | E1535*              | V272L              | WT                       |
| AR2  | Anastomosis | Frozen/gDNA | cDNA G13D      | –                                    | –              | E1535*              | V272L              | WT                       |
| 2    | PT       | Ascending colon   | FFPE/gDNA      | –                                    | –              | –                    | –                  | –                       |
| AR1  | Anastomosis | Frozen/gDNA | cDNA G13D      | –                                    | –              | –                    | –                  | –                       |

AR = anastomotic recurrence, cDNA = complementary DNA, FFPE = formalin-fixed paraffin-embedded, gDNA = genomic DNA, PT = primary tumor, WT = wild type, – = not performed.

the KRAS G13D mutation may be associated with AR due to the aggressive nature of the cancer cells. A study reported that patients with KRAS G13D-mutant tumors showed poor prognosis with standard chemotherapy alone or best supportive care.1,2,3 They also showed improved outcomes under treatment with cetuximab.4,5 This is a paradox, considering the reports that KRAS-mutant tumors are resistant to anti-epidermal growth factor receptor therapy.4,12 Another study also supported this result in that KRAS G13D mutations were associated with improved clinical outcomes when cetuximab was added to first-line chemotherapy.4,14 Our patients may have benefited from chemotherapy with cetuximab, although current guidelines do not recommend a cetuximab regimen for KRAS-mutant CRC, and thus health insurance in Japan does not cover this regimen. Several studies have reported a correlation between colorectal liver metastasis and KRAS mutation.6,15,16 However, no reports have demonstrated a relationship between AR and KRAS mutation. Further studies with larger sample sizes are warranted.

The terminology of AR is arguable, and distinguishing between AR and secondary PT development may be difficult. Gopalan et al. reviewed the literature and defined local recurrences and secondary PTs according to the duration between the development of anastomotic tumors and PTs.17 However, this definition is arbitrary. More theoretically, secondary PTs harbor distinct genetic mutations, whereas the mutations of an AR may be similar to those of the PT. In our cases, the lesions shared the same mutation profile, and genetically they were considered ARs.

There are several limitations to this study. First, the PT specimen in Case 2 (2-PT) was unavailable. In addition, the second AR specimen in Case 1 (1-AR2) could not yield sufficient DNA due to the chemoradiation effect. The present study investigated only 2 cases, and thus the number of lesions assessed was limited. However, despite the limited number of AR cases, we were able to clarify the genetic profile and determine potential relationships between genetic abnormalities and AR. Second, we did not perform comprehensive next-generation sequencing and only included key gene profiles. There may be novel mutations associated with AR.

Despite such limitations, the present case reports may provide some insights on the mechanism of AR in CRC, in that they showed that the PT harbored the same mutations as the AR, and the lesions in both cases harbored the KRAS G13D mutation.

Acknowledgments
The authors would also like to thank Editage (https://www.editage.jp/) for the English language review.

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