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Microsatellite alterations at selected tetranucleotide repeats are associated with morphologies of colorectal neoplasias

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Abstract

Background & Aims—Elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) occurs during microsatellite instability (MSI) that is not associated with major defects in DNA mismatch repair (MMR) but rather the reduced (heterogenous) expression of the MMR protein hMSH3; it occurs in sporadic colorectal tumors. We examined the timing of development of EMAST during progression of colorectal neoplasias and looked for correlations between EMAST and clinical and pathology features of tumors.

Methods—We evaluated tumor samples from a cohort of patients that had 24 adenomas and 84 colorectal cancers. EMAST were analyzed after DNA microdissection of matched normal and tumor samples using the polymorphic tetranucleotide microsatellite markers MYCL1, D9S242, D20S85, D8S321, and D20S82; data were compared with clinical and pathology findings. Traditional MSI analysis was performed and hMSH3 expression was measured.

Results—Moderately-differentiated adenocarcinomas and poorly-differentiated adenocarcinomas had higher frequencies of EMAST (56.9% and 40.0%, respectively) than well-differentiated adenocarcinomas (12.5%) or adenomas (33.3%) (*P*=0.040). In endoscopic analysis, ulcerated tumors had a higher frequency of EMAST (52.3%) than flat (44.0%) or protruded tumors (20.0%) (*P*=0.049). In quantification, all tumors with >3 tetranucleotide defects lost MSH3 (>75% of cells); nuclear

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heterogeneity of hMSH3 occurred more frequently in EMAST-positive (40.0%) than in EMAST-negative tumors (13.2%) (P=0.010).

Conclusions—EMAST is acquired during progression of adenoma and well-differentiated carcinomas to moderately and poorly differentiated carcinomas; it correlates with nuclear heterogeneity for hMSH3. Loss of hMSH3 corresponds with multiple tetranucleotide frameshifts. The association between EMAST and ulcerated tumors might result from increased inflammation.

Keywords

Colon cancer; mutation; DNA repair; cancer genetics

INTRODUCTION

Defects in DNA mismatch repair (MMR) cause microsatellite instability (MSI) and help direct subsequent development of malignancy in patients with Lynch syndrome (due to germline mutation of a DNA MMR gene), and in 15–20% of patients with sporadic colorectal cancers (CRC) (due to epigenetic inactivation of the DNA MMR gene hMLH1).[1–4] Loss of DNA MMR in these conditions manifest as frameshift mutations in sequences that contain mono-[e.g. $(A)_n$] or di-nucleotide [e.g. $(CA)_n$] repeats that loop during DNA replication. A separate form of MSI described in respiratory, genitourinary, and other tumors,[5–12] and previously not linked to defects in DNA MMR is termed elevated microsatellite alterations at selected tetranucleotide repeats (EMAST). EMAST is identified when tetranucleotide DNA sequences [e.g. $(AAAG)_n$ or $(CTTT)_n$] show frameshift mutations. In both MSI and EMAST alterations, frameshifts occur by the length of the repeat unit due to the nature of complimentary binding with the newly-synthesized strand. Most microsatellites are in non-coding region of DNA, but a small minority resides in genes that may drive the pathogenesis of CRC in particular, as frameshifts in these genes have functional growth consequences for cells.[2,13]

In a sentinel report, EMAST was observed in 60% of CRC and associated with heterogenous expression of the DNA MMR protein hMSH3, suggesting a causal link between loss of the hMSH3 repair profile and EMAST. While all classic MSI tumors in this report also demonstrated EMAST, most EMAST tumors were not MSI, indicating that EMAST spans multiple forms of genomic instability, and may be an acquired defect. Indeed, it has been suggested that an overlapping mechanism may exist between MSI-low (MSI-L), EMAST and loss of heterozygosity (LOH),[14] as all of these mechanisms have not been associated with inactivation of DNA MMR. The observation of EMAST in CRC begs the question of its timing for occurrence in the adenoma-carcinoma sequence given its finding in 60% of colorectal cancers, and what clinicopathological patterns might predict its presence. The linkage of timing and clinicopathological data to EMAST may indicate the consequences of EMAST upon tumor development while its cause is being evaluated. We therefore examined EMAST in adenomas and various staged adenocarcinomas to assess timing during colorectal neoplastic progress and correlated associated clinicopathological data with EMAST.

MATERIALS and METHODS

Tissue samples

We evaluated a cohort of 108 patients who have 24 adenomas and 84 colorectal cancers that were resected between 2008 and 2009 at Konkuk University Medical Center, Seoul, South Korea. All patients provided informed consent, and this study was approved by the Institutional Review Board of Konkuk University Medical Center, which confirmed that the study was in accordance with the ethical guidelines of the Helsinki Declaration. This study was also

approved by the Institutional Review Board on Human Subjects Protection at University of California San Diego.

Location, size, and shape of tumors were recorded at colonoscopy. Macroscopic features were divided into three major types: (i) protruded, elevated, or polypoid, (ii) sessile, superficial, or flat, and (iii) ulceroinfiltrative, depressed, or excavated (Figure 1). Tumors showing multiple macroscopic features were classified on the predominant subtype. When the tumor was suspected or diagnosed as adenoma, endoscopic resection was done by an endoscopist (Dr. S.-Y. Lee). When the tumor was suspected or diagnosed as cancer, it was referred for surgical resection by a surgeon (Dr. D.-Y. Hwang or M. K. Seong).

All specimens were diagnosed by a single pathologist (Dr. H. S. Han) to avoid interpersonal variability. Histopathology, width, height, and depth of the tumor were recorded. To differentiate between well-differentiated and moderately-differentiated adenocarcinomas, the following diagnostic criteria was used: (i) well-differentiated adenocarcinomas were diagnosed when gland-forming area encompasses more than 95%, (ii) moderately-differentiated between 50–95%, and (iii) poorly-differentiated between 5–49%, and undifferentiated < 5%. Evidence for lymphatic, venous and, perinureral invasion, and number of metastatic lymph nodes were recorded for colorectal cancers. TNM staging was recorded based on postsurgical findings.

Genomic DNA extraction, EMAST and MSI analysis

Serial 10 μm sections of tissues were cut from formalin-fixed and paraffin-embedded tissues and mounted on glass slides. Microdissection was performed to extract genomic DNA from tumor and counterpart normal tissue. Genomic DNA from microdissected tissues was isolated using Qiagen DNeasy Blood and Tissue Kit (Qiagen, USA) following the manufacturer's instruction. Briefly, one thousand μl of xylene was added to each eppendorf tube containing microdissected tissue, and the tubes were centrifuged at 14,000 rpm for 5 min at room temperature (RT). After removing the supernatant, 1000 μl of 100% ethanol was added, and all the tubes were centrifuged at 14,000 rpm for 5 min at RT. After removing the ethanol, the samples were incubated at 37°C for 15–25 minutes until the ethanol was evaporated. Tissues were incubated in lysis buffer solution containing 20 μl of proteinase K solution at 56°C overnight. On the following day, samples were treated with buffers according to the manufacturer's instructions, and were centrifuged at 8,000 rpm for 1 min twice and 14,000 rpm for 3 min. After incubating at room temperature for 5 min, DNA was extracted by centrifuging at 8,000 rpm for 1 min.

To determine EMAST, 5 polymorphic tetranucleotide markers (MYCL1, D9S242, D20S85, D8S321, and D20S82) were utilized. Genomic DNA extracted from tumors and counterpart normal tissues were PCR-amplified by specific primers for each tetranucleotide marker using Platinum® PCR Supermix (Invitrogen, USA) as per the manufacturer's protocol. The PCR products were used for DNA sequencing using an ABI 3700 analyzer to identify mutation at tetranucleotide repeats of each loci. Tumors showing frameshifts in at least 2 tetranucleotide markers were categorized as EMAST-positive tumors.

MSI analysis was performed using five mononucleotide markers (*BAT-25*, *BAT-26*, *NR-21*, *NR-24*, and *MONO-27*) and three dinucleotide markers (*D2S123*, *D17S250*, and *D5S346*) for MSI determination and two polymorphic pentanucleotide markers (*Penta C* and *Penta D*) for sample identification. We defined MSI-H when >30% of examined loci showed frameshifts, MSI—L when <30% but >0% of examined loci had frameshifts, and MSS (microsatellite stable) when no instability was present at any of the loci tested.

Immunohistochemistry for hMSH3

Slides containing colon tumor tissue were deparaffinized in xylene, and rehydrated in 100%, 95%, and 70% alcohols to water. The slides were immersed in sodium citrate buffer (pH 6.0) and heated in a microwave for 10 minutes for antigen retrieval. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2. Ten percent horse serum was added for 1 hour to block nonspecific protein binding. Rabbit hMSH3 antibody was incubated overnight at 1:800 dilution. Slides were then processed using a DAKO Signal Catalyzed Amplification System (DAKO Corp., Carpinteria, CA). Biotinylated anti-rabbit immunoglobulin G was added for 15 minutes, followed by incubation with peroxidase-labeled streptavidin for 15 minutes at room temperature. The sections were incubated with 3,3-diaminobenzidine tetrahydrochloride and H_2O_2 for 3 minutes, counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped. The percentage of blue basal nuclei stain among tumor cells was calculated as hMSH3 loss using a 100× objective lens. Nuclear heterogeneity was determined when there was both brown (stained) and blue (unstained) nuclear staining using a 400× objective lens. Slides were analyzed by a single pathologist who was unaware of the clinical, MSI, and EMAST results.

Statistical analysis

To study the statistical association between EMAST and other variables, continuous and categorical demographic data were compared by *t*-tests and chi-square tests, respectively. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

The frequency of EMAST increases in more advanced colorectal neoplasms

EMAST was determined when at least 2 loci showed frameshifts at tetranucleotide repeat loci compared to matched normal tissue. Overall, 50% of tumors from 108 patients possessing 24 adenomas and 84 colorectal cancers were EMAST-positive. Additionally, by standardized MSI analysis, there were 6 MSI-H, 10 MSI-L, and 92 MSS colorectal neoplasms among the 108 tumors (Table 1). All 6 MSI-H (100%) neoplasms were cancers, and all (100%) were EMAST-positive, whereas 7 of 10 MSI-L neoplasms (70%, all cancers) were EMAST-positive. All 24 adenomas were MSS.

To eliminate the possibility of MSI-H tumors cross-identifying EMAST tumors, we analyzed our cohort for EMAST after excluding the 6 MSI-H cases. Clinicopathological characteristics of 48 EMAST-positive tumor cases versus 54 EMAST-negative tumor cases are summarized in Table 1. There was no significant difference for location (p=0.638), shape (0.536), or size (p=0.392) between EMAST-positive and EMAST–negative adenomas. The proportion of EMAST-positive neoplasms increased with the advancement of histology. Moderately differentiated adenocarcinomas (56.9%) and poorly differentiated adenocarcinomas (40.0%) showed higher frequency of EMAST than well differentiated adenocarcinomas (12.5%) and adenomas (33.3%) (*p*=0.040). Individual details of the 40 EMAST-positive cancers are summarized in Table 2.

Higher frequency of EMAST in ulcerated colorectal tumors

EMAST-positive and -negative tumors cases were also categorized by endoscopic findings (Table 1). We found that downward growing ulcerated (depressed or excavated) tumors (52.3%) showed a significantly higher frequency for EMAST-positivity than sessile (superficial or flat) tumors (44.0%) and protruded (elevated or polypoid) tumors (20.0%) (*p*=0.049). In addition, of a total of 62 ulcerated cancers, regardless of growth patterns, 34

(54.8%) showed EMAST, whereas only 6 (37.5%) of 16 non-ulcerated cancers showed EMAST.

Relation of EMAST with tumor location and invasiveness of colorectal cancers

There was no significant preference for location of EMAST-positive tumors, as 42.8%, 41.3%, and 60.7% of EMAST was in proximal, distal, and rectal locations, respectively (Table 1). However, one EMAST locus, *Mycl1*, showed a significantly higher frequency (46.4%) of frameshifts in rectal tumors compared with proximal (21.4%) and distal (19.6%) colon tumors (p=0.030). The other 4 EMAST loci tested did not show any difference in frameshift frequency relative to the location of the neoplasms.

There was no significant association between EMAST and tumor microinvasion (venous invasion, perineural invasion, and lymphatic invasion) among the 84 colorectal cancers. Venous invasion was noticed in 4 of 40 EMAST-positive cancers (10.0%) and in 1 of 38 EMAST-negative cancers (2.6%), respectively (p=0.359). However, the EMAST locus *Mycl1* was more frequently frameshifted in cancers with venous invasion (80.0%) than cancers without venous invasion (27.4%) (p=0.029). Additionally, perineural invasion was noticed in 6 of 40 EMAST-positive cancers (15.0%) and in 4 of 38 EMAST-negative cancers (10.5%), respectively (p=0.738). On the other hand, lymphatic invasion was noticed in 8 of 40 EMAST-positive cancers (20.0%) and in 8 of 38 EMAST-negative cancers (21.1%), respectively (p=1.000). The EMAST marker D20S82 was less frequently observed in cancers with lymphatic invasions (12.5%) than cancers without lymphatic invasion (45.2%) (p=0.021).

None of the T, N, M stages correlated with the presence of EMAST. The frequency of EMAST was 40% in Tis, 50% in T1, 33.3% in T2, 58.0% in T3, and 42.9% in T4 (p=0.574). EMAST prevalence according to N staging was 51.3% in N0, 45.5% in N1, 58.8% in N2 (p=0.710). Additionally, there was no significant difference in EMAST frequency between cancers with metastasis (54.5%) and without metastasis (44.2%) (p=1.000).

Loss of hMSH3 expression and heterogeneity

Most tumors demonstrated nuclear heterogeneity by expressing both brown (positive) and blue (negative) nuclei upon hMSH3 immunohistochemical staining (Figure 2). This nuclear heterogeneity was noticed in 16 of 40 EMAST-positive cancers (40.0%), whereas it was detected only in 5 of 38 EMAST-negative cancers (13.2%) (p=0.010). Quantitatively, all tumors with >3 tetranucleotide defects showed significant hMSH3 loss (blue nuclei exceeding 75% of all stained basal nuclei in tumor cells), whereas this degree of hMSH3 loss was rare in tumors with no tetranucleotide defects (Figure 3).

DISCUSSION

In this study, we examined the frequency of EMAST in colorectal neoplasms and its linkage to clinicopathological variables, as there is no data for EMAST in this regard for CRC. We discovered that EMAST is less frequent in colorectal adenomas and well differentiated adenocarcinomas than in moderately differentiated and poorly differentiated adenocarcinomas, suggesting that EMAST is progressively acquired during the histological adenoma-carcinoma sequence, from adenoma to well-differentiated carcinomas to moderately and poorly differentiated carcinomas. CRC progresses genetically through at least two pathways involving genomic instability: chromosomal instability (CIN) and MSI, with the latter phenotype caused by loss of DNA MMR. Although it is still not clear whether EMAST just represents bystander mutations in a large subset of tumors with specific defects in DNA replication and repair, the lower EMAST prevalence in adenomas in our study suggests that the adenoma-carcinoma sequence is related to the development of EMAST.

In defining MSI originally,[1] some tumors were characterized as MSI-L and not associated with loss of DNA MMR, the cause of MSI-H. This definition is predicated on utilizing mono and dinucleotide markers, and not tetranucleotide markers.[1] Longer repeats in microsatellites that frameshift are characteristically caused by loss of hMSH3 function, based on bacteria and gene data, as this DNA MMR protein helps repair longer insertion-deletion DNA loops during DNA replication.[2] The finding of heterogenous expression of hMSH3 in CRC that are EMAST-positive seems to confirm hMSH3 as a potential cause of EMAST. Could tumor progression be associated with loss of hMSH3? This question has not been answered and may be somewhat difficult given the heterogenous nature of hMSH3 loss in colorectal tumors.[15] Our finding strongly indicates that EMAST is an acquired feature of CRC. Additionally, our data showing nuclear heterogeneity for hMSH3 expression among EMAST-positive tumors, plus the quantitative association of the number of tetranucleotide frameshifts linked to hMSH3 loss strongly suggest that hMSH3 loss is linked to EMAST-positive neoplasm.

We found that EMAST is more frequent in colorectal tumors with ulcerated features. We speculate that EMAST association with ulcerated cancers may be a consequence of increased local inflammation. Colorectal tumors develop an increasing level of inflammatory infiltration during progression from adenoma to cancer, and would be highest in cancers in which mucosa is breached as in ulcerated cancers. Endoscopically, colon tumors grow in three different ways; upward, downward, and lateral.[16] Of these three types, depressed lesions are most aggressive and inflammatory due to its ulceroinfiltrative nature. This is consistent with our result that tumors with downward growth showed higher prevalence of EMAST than upward or lateral growth tumors. In addition, cancers with ulceration showed higher prevalence of EMAST than cancers without ulceration.

It is possible that inflammation may drive EMAST. First, samples from inflamed colons from ulcerative colitis patients demonstrate MSI-L without major DNA MMR defects, but were not specifically tested for loss of hMSH3 expression.[17] Second, *in vitro* experiments indicated that oxidative stress can “relax” or impair DNA MMR function, and can cause MSI.[18,19] Thus, one hypothesis is that during the adenoma-carcinoma progression, EMAST is generated by progressive loss of hMSH3, for instance, due to inflammatory-induced impairment of this protein's function. As indicated in our study, hMSH3-positive and -negative cells were interspersed in most of the tumors, with nuclear heterogeneity being more common in EMAST-positive cancers than in -negative cancers. This suggests the possibility of hMSH3-negativity being a reversible trait as mentioned by Haugen et al.[15] In our study, cancers with an ulcerative appearance showed a higher number of tetranucleotide defects, and all cancers with >3 tetranucleotide defects showed severe loss of hMSH3 expression. Our results are consistent with a hypothesis that the inflammatory process in ulceroinfiltrative cancers might be related to down-regulation of hMSH3 expression, and as a consequence demonstrate a higher number of tetranucleotide defects. In addition, recent evidence suggests EMAST is associated with increased numbers of inflammatory cells infiltrating a tumor.[20]

In regard to the invasiveness of cancers, there was no correlation between EMAST and TNM stages as well as with venous, perineural, or lymphatic invasions. Similarly, there was no significant difference in EMAST prevalence based on tumor location in the colon. This suggests that these parameters occur independent of EMAST, which is in contrast to classic MSI-H, which is associated with a right colon location, earlier cancer stage, and less tumor invasion. It is also possible that utilizing 2 of 5 teranucleotide frameshifts as the definition of EMAST might not count some EMAST tumors, as some have defined EMAST-positivity when 1 of 5 markers show a frameshift. In this study, if we defined EMAST as 1 of 5 defective tetranucleotide markers, the overall prevalence of EMAST increased from 50% to 88.9% (96/108), a prevalence higher than in previous reports.[14,15,20] Given the association of consistent hMSH3 loss with >3 tetranucleotide frameshifts in our study, a frequency higher

than one tetranucleotide defect will likely be more appropriate to define EMAST as new data is produced. Despite this, it is still possible that some EMAST-positive tumors might have been ignored by using our definition of EMAST, since 5 of 54 EMAST-negative tumors showed hMSH3 heterogeneity by immunohistochemistry. A large-scale study may help determine the detection sensitivity of EMAST-positive tumors in relationship to hMSH3 expression.

In summary, EMAST is less frequent in colorectal adenomas, well differentiated adenocarcinomas, and colorectal cancers without ulcerations. EMAST is associated with nuclear heterogeneity of hMSH3 expression. These observations suggest that the EMAST phenotype is linked to adenoma-to-carcinoma morphology as well as the endoscopic appearance of ulceration, and nuclear hMSH3 expression.

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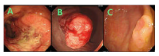

Figure 1. Endoscopic appearance of tumors

Tumors were subclassified into: **(A)** ulceroinfiltrative, depressed, or excavated tumors, **(B)** protruded, elevated, or polypoid tumors, and **(C)** sessile, superficial, or flat tumors.

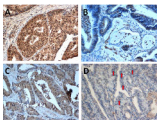

Figure 2. hMSH3 expression patterns by immunohistochemistry

(A) An hMSH3-positive (brown nucleus staining) tumor. (B) An hMSH3-negative (blue nucleus staining) tumor. Most EMAST tumors showed (C) a mixed appearance with partial hMSH3 loss. (D) Heterogeneity was diagnosed when there is a nucleus showing both brown and blue staining in the nucleus (arrows).

Figure 3. Degree of hMSH3 loss according to the number of tetranucleotide frameshifts among 78 cancers

Each black circle indicates a tumor with heterogeneity in the nucleus, whereas white circles indicate a tumor without heterogeneity. Tumors with >3 tetranucleotide defects showed the highest percentage of hMSH3 loss.

Table 1

Correlation of EMAST and clinicopathological features

	EMAST-positive tumors (n=48)	EMAST-negative tumors (n=54)	*p*-value
Age (mean±SD, year-old)	60.31 ± 12.97	62.09 ± 11.40	0.462
Gender (male : female)	29 : 19	36 : 18	0.542
Pathology			**0.040**
Adenoma	8 (16.7%)	16 (29.6%)	
Well differentiated adenocarcinoma	1 (2.1%)	7 (12.9%)	
Moderately differentiated adenocarcinoma	37 (77.1%)	28 (51.9%)	
Poorly differentiated adenocarcinoma	2 (4.2%)	3 (5.6%)	
Endoscopic findings			**0.049**
Upward growth (protruded, elevated, or polypoid)	3 (6.3%)	12 (22.2%)	
Lateral growth (sessile, superficial, or flat)	11 (22.9%)	14 (25.9%)	
Downward growth (depressed, excavated, or ulceroinfiltrative)	34 (70.8%)	28 (51.9%)	
Location			0.234
Proximal colon	12 (25.0%)	16 (29.6%)	
Distal colon	19 (39.6%)	27 (50.0%)	
Rectum	17 (35.4%)	11 (20.4%)	

EMAST, elevated microsatellite instability at selected tetranucleotide repeats.

Table 2

Status of tetranucleotide marker defects, microsatellite instability, and hMSH3 expression in 40 EMAST-positive cancers

No	Diagnosis	Shape	MYCL1	D9S242	D20S85	D8S321	D20S82	MSI result	hMSH3 loss (%)
39	MD	Ulcerative	○				○	MSS	6
113	MD	Ulcerative	○			○		MSS	7
183	MD	Ulcerative	○			○		MSS	8
205	MD	Ulcerative			○	○		MSS	8
117	MD	Ulcerative	○	○				MSS	11
171	MD	Ulcerative	○			○		MSS	15
9	MD	Polypoid	○	○				MSS	18
75	MD	Ulcerative				○	○	MSS	27
165	MD	Ulcerative	○	○				MSS	30
181	MD	Ulcerative		○		○		MSS	32
187	MD	Ulcerative	○			○		MSS	33
175	PD	Ulcerative				○	○	MSS	38
11	MD	Ulcerative	○			○		MSS	60
15	MD	Ulcerative			○	○		MSS	94
65	MD	Ulcerative		○			○	MSS	95
23	MD	Sessile				○	○	MSS	95
55	MD	Ulcerative		○			○	MSS	99
153	MD	Ulcerative		○		○		MSS	99
121	MD	Ulcerative	○				○	MSS	99
107	MD	Ulcerative	○		○			MSS	99
89	MD	Ulcerative			○	○		MSI-L	2
207	MD	Ulcerative			○	○		MSI-L	61
77	PD	Ulcerative		○		○		MSI-L	62
61	MD	Ulcerative		○	○			MSI-L	100
7	WD	Sessile		○	○	○		MSS	4
147	MD	Ulcerative		○		○	○	MSS	10
57	MD	Polypoid			○	○	○	MSS	14

No	Diagnosis	Shape	MYCL1	D9S242	D20S85	D8S321	D20S82	MSI result	hMSH3 loss (%)
47	MD	Ulcerative		○	○		○	MSS	26
85	MD	Ulcerative		○		○	○	MSS	29
93	MD	Ulcerative	○			○	○	MSS	30
103	MD	Ulcerative	○		○		○	MSS	59
115	MD	Ulcerative	○		○		○	MSS	88
97	MD	Ulcerative	○		○		○	MSS	89
33	MD	Polypoid		○	○		○	MSS	98
71	MD	Ulcerative		○		○	○	MSS	99
17	MD	Ulcerative	○	○		○		MSI-L	51
53	MD	Ulcerative		○		○	○	MSI-L	100
27	MD	Polypoid	○	○		○	○	MSI-L	94
41	MD	Ulcerative	○	○	○		○	MSS	98
141	MD	Ulcerative	○	○	○	○	○	MSS	99

WD, well-differentiated adenocarcinoma; MD, moderately-differentiated adenocarcinoma; PD, poorly-differentiated adenocarcinoma; MSI-L, microsatellite instability-low; MSS, microsatellite stable. ○ indicates tetranucleotide frameshift defect.