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MSH6 immunohistochemical heterogeneity in colorectal cancer: comparative sequencing from different tumor areas

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Summary

Mismatch repair protein (MMR) immunohistochemistry is an important tool in screening for Lynch syndrome in colorectal cancer patients. Unusual staining patterns such as heterogeneous MSH6 staining have been reported in colorectal and endometrial cancers. We aim to better understand MSH6 staining heterogeneity in colorectal cancer by comparative sequencing of different tumor areas for MMR and DNA polymerase mutations. Whole-section slides of 1754 colorectal cancers were reviewed for heterogeneous MSH6 staining, defined as discrete tumor areas with abrupt loss of staining juxtaposed to tumor areas with retained staining. Nine cases (0.05%) demonstrated heterogeneous MSH6 staining; none received neoadjuvant therapy prior to the specimen collection. The area of tumor with loss of MSH6 expression ranged from 5% to 60% (average 22%). Four cases had enough tissue remaining in both retained and lost MSH6 areas to perform tumor sequencing on both areas. All 9 cases were negative for *MSH6* germline mutation; MSH6 heterogeneous staining was seen in tumors with *MLH1* or *PMS2* abnormalities (6 cases of *MLH1* methylation, 2 *PMS2* germline mutation, 1 *MLH1* germline mutation). In addition, case 1 also had a somatic *POLD1* exonuclease domain mutation (p.Y405C) in the MSH6 loss area but not in the intact area. We recommend reporting MSH6 heterogeneous pattern as MSH6 staining is present with a comment stating that the heterogeneous pattern typically does not indicate germline mutation in *MSH6* but is commonly associated with abnormality in another MMR gene such as *MLH1* or *PMS2*, or even other DNA repair genes such as DNA polymerase.

Keywords

Colorectal cancer; MSH6 immunohistochemistry; Heterogeneous staining; DNA polymerase; c.3261

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1. Introduction

Mismatch repair protein (MMR) immunohistochemistry is a widely adopted tool for universal tumor screening for Lynch syndrome in newly diagnosed colorectal and endometrial cancer patients. Identification of patients with MMR deficient tumors can also indicate which patients might benefit from immunotherapy [1]. The interpretation of MMR immunohistochemistry in most cases is straightforward, showing either diffuse strong staining or complete loss of staining in tumor cells. However, occasional cases with patchy/heterogeneous MMR staining exist. In general daily practice, most pathologists regard such patchy/heterogeneous staining that is as strong as control as retained expression.

A distinct heterogeneous MMR staining pattern has been described that is characterized by tumor areas with abrupt loss of staining juxtaposed to tumor areas with retained staining. This pattern can be described as subclonal loss of staining. Importantly, the control staining is intact in all areas. This abrupt or subclonal loss of staining has so far been most often observed with MSH6 staining in colorectal cancers (CRCs) [2–7] and few endometrial cancer cases [3,8]. In contrast, MLH1 heterogeneous staining has been mostly reported in endometrial cancer due to heterogeneous *MLH1* promoter methylation [8,9], although few CRC cases with this pattern were also reported [4,5,7].

Possible explanations for the heterogeneous staining pattern include (1) variable epitope expression; (2) heterogeneous second-hit mutation (germline or somatic mutations with the second hit in part of the tumor but not all); (3) accumulation of *MLH1* methylation in select tumor areas [9]; (4) post neoadjuvant therapy [4,10–13]; and/or (5) preanalytical variables (poor fixation, tissue hypoxia, oxidative stress [4,13]) and antibody technical issues. In the clinical setting of Lynch syndrome screening, it is vital to understand how to interpret the heterogeneous staining and determine if this subclonal loss of staining suggests a heterozygous germline mutation with focal second-hit mutation (Lynch syndrome), subclonal epigenetic silencing/other secondary changes (sporadic tumor), or something else (preservation/technical/treatment-related artifacts). In addition, studies have shown that mutation(s) of other DNA damage repair genes such as DNA polymerase (exonuclease domain) and *MUTYH* can cause MMR deficiency phenotype due to DNA damage repair errors [14–17].

Although a few studies have reported heterogeneous MMR staining, only limited data are available directly comparing molecular alterations between the staining lost versus retained areas. In addition, genetic alterations of DNA polymerase have not been examined in the context of heterogeneous MMR staining. In this study, we aim to study MMR molecular alterations in CRCs with heterogeneous MSH6 staining by selective sampling and comparative sequencing of MMR and DNA polymerase genes from tumor areas with and without MSH6 staining.

2. Materials and Methods

A total of 3346 newly diagnosed CRC patients were prospectively enrolled in Ohio Colorectal Cancer Prevention Initiative (OCCPI, [ClinicalTrials.gov](https://clinicaltrials.gov) identifier:

[NCT01850654](#); January 1, 2013 to December 31, 2016). Institutional Review Board approval and written informed consent were obtained. Methods for the OCCPI have been described previously [18,19], but briefly, 3310 patients successfully underwent MMR immunohistochemical (IHC) testing and/or microsatellite instability (MSI) by polymerase chain reaction testing (Promega MSI Analysis System [Version 1.2], Madison, WI), which includes 5 repeat markers (BAT-25, BAT-26, NR-21, NR-24, MONO-27). Tumors with 2/5 markers showing instability were classified as MSI-high (MSI-H). Immunohistochemistry of the MMR proteins was performed using the 2-stain method [20]. Staining for all 4 MMR proteins was done as routine clinical care for some patients and attempted for all patients if MSI could not be performed or if the MSI and 2-stain IHC results were discordant. Staining performed at The Ohio State University Wexner Medical Center used the following commercial antibodies: MLH1 (Clone ES05; Leica/Novacastra, Buffalo Grove, IL), PMS2 (Clone A16-4; BD Pharmingen, San Jose, CA), MSH2 (Clone FE11; Calbiochem, Basel-Land, Switzerland), MSH6 (Clone EP49; Epitomics, Burlingame, CA), and PMS2 (Clone: BD Pharmingen A16-4, mouse: 556415). *MLH1* methylation was done using pyrosequencing [21] when tumors were MSI-H and/or when MLH1 and PMS2 proteins were absent on IHC, with 15% methylation classified as positive.

For this study, all cases with available MMR stained whole-section slides (n = 1754) were retrospectively reviewed for heterogeneous MSH6 staining. *Heterogeneous staining* was defined as a patchy staining pattern with discrete/subclonal areas of tumor that show abrupt loss of MSH6 staining (confluent areas of staining loss involving multiple adjacent glands) next to areas with retained staining, in the presence of appropriately stained internal control cells in both areas. The percentage of staining loss area was noted for each heterogeneous staining case. Cases with nonabrupt patchy staining intensity (vague regional variation) that was as strong as control were excluded, as these may be attributed to suboptimal staining related to preanalytical factors, antibody technical issues, or neoadjuvant treatment effect [4,8,10,11,13,22,23]. Cases with control failure were discarded if repeat staining was not successful. Nine cases with heterogeneous staining of MSH6 were identified, and none were previously treated with neoadjuvant chemoradiation.

For the OCCPI study, patients with MMR deficiency without *MLH1* methylation underwent germline next-generation sequencing (NGS) panel testing (ColoSeq or BROCA, University of Washington, Seattle, WA). Genomic regions were captured using biotinylated RNA oligonucleotides (SureSelect, Agilent Technologies, Santa Clara, CA) and sequenced on an Illumina HiSeq2000 instrument (Illumina, San Diego, CA) [24]. Large rearrangements were detected [25]. Tumor sequencing with ColoSeq Tumor of the MMR genes followed for patients with unexplained MMR-deficient tumors. Data were created by the NGS Laboratory and Analytics group (Seattle, WA).

For this study, 4 of the 9 cases had enough tissue remaining in both retained and lost MSH6 areas to perform additional tumor sequencing (cases 1 to 4 in the Table). For each case, the pathologist (W. C.) marked discrete tumor areas with intact MSH6 expression and MSH6 loss. Tumor macrodissection was performed by scraping the corresponding areas of tissue from unstained glass slides, and the scrapings were collected in separate tubes. Tumor DNA was then extracted from the retained and lost staining areas, respectively, for subsequent

sequencing. MSI was confirmed by mSING method [26] in both tumor areas. The 6 patients with *MLH1* promoter hypermethylation received germline NGS of the MMR genes at InVita (San Francisco, CA). The 3 patients with abnormal staining without *MLH1* methylation received germline NGS of the MMR genes at the University of Washington.

3. Results

3.1. Patient characteristics

A total of 1754 colorectal cases with available MMR stained whole-section slides were retrospectively reviewed for heterogeneous MSH6 staining. Nine of 1754 CRC cases (0.05%) demonstrated heterogeneous MSH6 staining featuring abrupt loss of MSH6 expression in areas of the tumor. The 9 patients' age ranged from 60 to 83 years (average 69 years). Female to male ratio is 7:2. Tumor site and tumor stage data are shown in the Table. None of these 9 patients had received neoadjuvant therapy prior to the specimen collection. For case 8 (rectal cancer), the specimen tested was tumor biopsy prior to any neoadjuvant treatment.

3.2. Tumor histology and MMR immunohistochemistry

Nine of 1754 CRC cases (0.05%) demonstrated heterogeneous MSH6 staining featuring abrupt loss of MSH6 expression in areas of the tumor. This heterogeneous staining pattern is demonstrated in a poorly differentiated carcinoma with large area of abrupt loss (Fig. 1A–C, case 2). In contrast, a case with nonabrupt patchy staining intensity was considered regional variation of staining (Fig. 1D–F) and was excluded. Two other cases of moderately differentiated adenocarcinoma showed a more interdigitated heterogeneous pattern with smaller areas of staining loss; nevertheless, the loss area was distinct and confluent involving multiple adjacent glands and thus considered true heterogeneous staining (Fig. 2A–F, case 3; Fig. 2G–L, case 5). The percentage of tumor area with MSH6 loss ranged from 5% to 60% (average 22%). In our cases with heterogeneous MSH6 staining, tumor grade was moderately differentiated (n = 8) and poorly differentiated (n = 1). No significant histomorphologic difference was noted when the areas with intact staining were compared to those with loss of staining. The control staining was intact and comparable in all areas of the tumors evaluated.

The expression of other MMR proteins (MLH1, PMS2, and MSH2) by immunohistochemistry mostly reflects the molecular status of corresponding genes (Table), except case 4 which demonstrated retained PMS2 staining despite having a likely pathogenic germline *PMS2* mutation (c.319CNT, p.R107W missense mutation). Possible explanations for retained PMS2 staining could be retained antigenicity with loss of function mutation due to the missense mutation, or the germline *PMS2* mutation could just be an incidental finding (without second hit/loss of function; therefore, not the cause of the CRC). Interestingly, case 3 showed accompanying MSH2 staining reduction/loss corresponding to the subclonal loss areas of MSH6 (Fig. 2A–F), whereas case 5 showed a minimally decreased MSH2 staining intensity in the corresponding area (Fig. 2G–L).

3.3. Molecular characteristics

All 9 MSH6 heterogeneous staining cases harbored molecular alterations in an MMR gene, and all were negative for germline mutations in *MSH6*. Six of 9 (67%) cases had *MLH1* promoter hypermethylation. One case (11%) harbored a germline *MLH1* pathogenic mutation (exon 16-19 deletion). Two cases (22%) were found to have germline *PMS2* pathogenic (exon 8 deletion) or likely pathogenic (c.319CNT, p.R107W) mutations.

Four cases (cases 1-4, Table) that had enough tumor tissue remaining in both retained and lost MSH6 areas underwent tumor microdissection and tumor sequencing. MSI was evaluated by mSINGS [26] method and was high in both tumor areas for all 4 cases. The finding that MSH6IHC retained areas were MSI-H is most likely due to *MLH1* hypermethylation or *MLH1* or *PMS2* germline mutation, and not the MSH6 alterations/heterogeneous loss. *BRAF*V600E mutation was present in both tumor areas for cases 1 and 3, consistent with *MLH1* hypermethylation status in these 2 cases. *BRAF*V600E mutation was absent in both tumor areas for cases 2 and 4. Somatic alterations of MMR genes (*MLH1*, *PMS2*, *MSH2*, *MSH6*, *EPCAM*), *POLE*, and *POLD1* in both tumor areas were compared and listed when present (Table).

In 2 of 4 cases (cases 1 and 3), double somatic mutation of *MSH6* was found in the MSH6 staining lost area but not in the retained area, as expected. In case 2, whereas the MSH6 intact area had 3 *MSH6* somatic mutations, all of them were at lower variant allele frequencies (VAFs; 0.17 and 0.19) than what is expected in the context of tumor content (estimated at 70%) if these mutations were in fact causing biallelic *MSH6* inactivation. In comparison, the MSH6 loss area had 1 somatic mutation with loss of heterozygosity (VAF 0.65, tumor content estimated at 60%) which definitely would explain the loss of MSH6 in that area of the tumor. Case 4 did not show difference in MSH6 somatic mutations between MSH6 intact and lost areas in the context of tumor content.

Of note, in case 1, a somatic *POLD1* exonuclease domain mutation (p.Y405C) and tumor ultrahypermutation were found only in the MSH6 loss area, not the intact area. There was no obvious *POLE* or *POLD1* mutation in either region in cases 2, 3, and 4.

4. Discussion

The interpretation of the MMR protein expression is generally straightforward in screening CRC for MMR deficiency, but as we continue to evaluate additional cases, unusual patterns are becoming more apparent. These less common patterns can lead to confusion, additional expense, and anxiety for clinicians and patients. It is important for pathologists to not immediately attribute these patterns to failed staining and ignore or suggest sometimes expensive additional testing. Many times, there are molecular explanations that help determine whether additional testing is necessary. *Heterogeneous expression* of MSH6 is defined as a patchy staining pattern with abrupt/subclonal loss of MSH6 staining in tumor areas juxtaposed to areas with retained staining, in the presence of appropriately stained internal control cells in both areas.

Our study demonstrates that heterogeneous MSH6 expression in CRC is a rare event (0.05%, 9 of 1754), comparable to prior studies of 0.003% (1 of 389 CRCs, McCarthy et al [5]; 3 of 1003 CRCs, Watson et al [7]), 2% (9 of 420 CRCs, Shia et al [12]), and 0.17% (22 of 13100, colorectal and endometrial carcinomas and 1 sebaceous neoplasm, Graham, et al [3]). It should be noted that the areas of MSH6 loss versus retention are distinct in our study cases, and the percentage of subclonal loss in each case differs, ranging from 5% to 60%. This is comparable with what was reported by Graham et al [3] (lost area ranging from <5% to 80%). Some have reported that a subset of CRCs [3] and endometrial cancers [9] has morphologic dedifferentiation (sheet-like growth, mucinous or signet ring cell features) in the area with staining loss, suggesting that the subclonal loss is a secondary event and a sign of tumor progression. However, we did not identify such dedifferentiation in our study, possibly because of the small sample size.

To the best of our knowledge, this study represents the largest series of CRCs with heterogeneous MSH6 staining that underwent MMR and DNA polymerase sequencing to compare the areas of tumor with MSH6 retained and lost. In 3 (cases 1, 2, and 3) of the 4 cases that were amenable for sequencing, the differences in somatic mutations of the *MSH6* gene between MSH6 intact and loss areas could explain the differential MSH6 expression pattern. However, the *MSH6* somatic mutations in case 4 cannot explain the heterogeneous staining. The most common *MSH6* somatic alteration is at the genomic locus c.3261 (3 of 4 cases, 1 case with c.3261del, p.F1088Sfs*2; 1 case with c.3261dup, p.F1088Lfs*9; and 1 case with c.3260_3261dup, p.F1088Pfs*3). The recent article by McCarthy et al [5] also reported similar finding in 2 CRC cases with heterogeneous staining.

Importantly, none of these cases with heterogeneous MSH6 staining were found to harbor germline *MSH6* abnormalities. This is in agreement with prior studies that demonstrated heterogeneous MSH6 staining is due to a sporadic deficiency secondary to another MMR abnormality. In our cohort, subclonal loss of MSH6 expression is either secondary to *MLH1* hypermethylation (67%, 6 of 9 cases) or a mutation of another MMR gene (2 cases with germline *PMS2* mutation and 1 case with germline *MLH1* mutation). It was first hypothesized by Yamamoto et al [27] and Yin et al [28] that a secondary mutation in the *MSH6* gene in its unstable mononucleotide tract could be induced by a mutation within another MMR gene. This hypothesis has since then been substantiated by studies in CRC, endometrial cancer, and sebaceous neoplasm [2–9].

Interestingly, in case 1, a somatic *POLD1* mutation in the exonuclease domain (p.Y405C) and resultant ultrahypermutation were identified in the MSH6 loss area but not in the MSH6 intact area. Mutation in the exonuclease domain of DNA polymerase *POLE* or *POLD1* may lead to impaired proofreading during DNA synthesis, resulting in a dramatically increased mutation rate. This has been associated with a subset of polyposis syndrome, CRC, and endometrial cancer [16,29,30]. Of note, somatic MMR deficiency has been reported in CRC with DNA polymerase mutation [17,31]. Given that *POLD1* somatic mutation was only identified in the MSH6 loss area but not the MSH6 intact area and that 3 somatic *MSH6* mutations were found in the MSH6 loss area but none in the MSH6 intact area, it is possible that the *MSH6* somatic mutations are secondary to *POLD1* and/or *MLH1* hypermethylation in this case.

A peculiar decreased/lost expression of MSH2 in the tumor areas that have subclonal MSH6 loss was found in 2 of the 9 cases (cases 3 and 7). Such MSH2 subclonal reduction has been previously noted in 1 endometrial cancer case [8] and some MSH6 heterogeneous staining cases by Graham et al [3]. This observation appears to challenge the dogma that the expression and staining of MSH2 should not be impacted by MSH6 status because MSH2 can also partner with MSH3 to avoid degradation [32,33]. Of note, the quantity of MutS α (MSH2 + MSH6) heterodimers outnumbers MutS β (MSH2 + MSH3) heterodimers [34,35], and it is likely that the loss of MSH6 expression decreases the stability of MSH2 due to the limited number of MSH3 available to dimerize with MSH2 proteins. Nevertheless, the finding of subclonal reduction in MSH2 can serve as a helpful clue in recognizing heterogeneous MSH6 staining.

Our study highlights the importance of identifying abrupt/subclonal loss of MSH6 staining, as such finding most likely represents secondary alteration of *MSH6* but not a germline mutation. Familiarity with this pattern could help avoid additional costly workup/anxiety for *MSH6* germline mutation in some cases; however, this pattern should not be dismissed as no abnormality or artifact. The presence of heterogeneous MSH6 pattern seems to be caused by somatic mutation of the unstable polycytosine tract in exon 5 within *MSH6* gene [3] secondary to another MMR abnormality (most commonly *MLH1* hypermethylation, followed by *MLH1* or *PMS2* mutation). Complete loss of MSH6 staining can also occur with *MLH1*/*PMS2* loss [2,7], which is presumed to occur by the same mechanism as described for heterogeneous loss. A limitation of this study is the small number of cases (4) that had enough tissues to study in MSH6 intact and lost areas; future studies with more cases may be helpful.

5. Conclusions

In our cohort, MSH6 heterogeneous staining is seen in tumors with *MLH1* or *PMS2* deficiency without *MSH6* germline mutation, indicating that this could be due to somatic *MSH6* mutations caused by other deficient MMR due to *MLH1* methylation or germline pathogenic variants in other MMR genes and/or somatic mutations in DNA polymerase genes. We recommend reporting MSH6 heterogeneous pattern as MSH6 staining is present with a comment stating that the heterogeneous pattern typically does not indicate germline mutation in *MSH6* but is commonly associated with abnormality in another MMR gene such as *MLH1* or *PMS2*, or even other DNA repair genes such as DNA polymerase.

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Competing interests: Ms. Hampel is on the scientific advisory board for InVita Genetics and Genome Medical; has conducted collaborative research with Myriad Genetics Laboratories, Inc, Ambry Genetics, and InVita Genetics; and has stock in Genome Medical. Ms. Pearlman has done collaborative research with Ambry Genetics, Myriad Genetics Laboratories, Inc, and InVita Genetics. Funding/Support: This study was supported in part by grant P30 CA016058 from the National Cancer Institute, Bethesda, MD.

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Fig. 1.

MSH6 staining in CRC: heterogeneous staining versus regional variability. A-C, Case 2: A poorly differentiated cecal cancer with *MLH1* promoter methylation showed heterogeneous MSH6 staining. Low (A, original magnification $\times 10$) and intermediate (B, $\times 100$) powers demonstrated abrupt area of staining loss (right) juxtaposed to area with retained staining (left). Notice positive-staining tumor-infiltrating lymphocytes as positive internal control (C, arrows, $\times 200$). D-F, In contrast, MSH6 staining with regional variability in staining intensity without confluent area of staining loss involving multiple adjacent glands was considered intact staining, possibly due to preanalytical factors and antibody technical issues (D, $\times 10$; E, $\times 100$; F, $\times 200$).

Fig. 2.

Additional examples of heterogeneous MSH6 staining in CRC. A-F, Case 3. A cecal cancer with *MLH1* promoter methylation showed loss of MLH1 staining (A, $\times 40$; B, $\times 200$) and heterogeneous staining pattern with MSH6 (E, $\times 40$; F, $\times 200$). Notice that MSH2 showed significantly reduced staining (weaker than internal positive control) in areas of MSH6 loss (C, $\times 40$; D, $\times 200$). G-L, Case 5. A transverse colon cancer with *MLH1* promoter methylation demonstrated loss of MLH1 staining (G, $\times 40$; H, $\times 200$) and heterogeneous staining pattern with MSH6 (K, $\times 40$; L, $\times 200$). Notice that MSH2 showed subtly reduced

but still intact staining in areas of MSH6 loss (I, $\times 40$; J, $\times 200$). PMS2 staining in both cases was lost as expected (not shown).

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Table

Clinicopathological features of CRCs with MSH6 heterogeneous staining.

#	Age (y)	Sex	Tumor site	pTNM	MSH6 lost area	MSH2	MLH1	PMS2	MLH1 methylation ^a	Germline mutation	MMR and POLE/POLD1 genetic alterations in tumor areas with lost MSH6 staining	MMR and POLE/POLD1 genetic alterations in tumor areas with retained MSH6 staining
1	75	F	N/A	T2cN0	30%	P	A	A	Yes	No ^b	<i>POLD1</i> : p.Y405C, VAF 0.26 <i>MSH6</i> : 3 somatic mutations (c.3522del, p.F1174Lfs*10, VAF 0.25; c.3312del, p.F1104Lfs*11, VAF 0.18; c.4001+2TNG, VAF 0.25) <i>MSH2</i> : 1 somatic mutation (c.299TNG, p.V100G, VAF 0.18)	<i>MSH6</i> : no mutation <i>MSH2</i> : 1 somatic mutation (c.299TNG, p.V100G, VAF 0.11)
2	68	F	Cecum	T2 N0	40%	P	A	A	Yes	No ^b	<i>MSH6</i> : 1 somatic mutation with LOH (c.3261del, p.F1088Sfs*2, VAF 0.65)	<i>MSH6</i> : 3 somatic mutations (c.3261del, p.F1088Sfs*2, VAF 0.17; c.3209del, p.G1070Vfs*9, VAF 0.19; 46-bp deletion, chr2:48030594-48030641)
3	60	F	Cecum	T3 N0	10%	H	A	A	Yes	No ^b	<i>MSH6</i> : 2 somatic mutations (c.1815_1816del, p.K606Nfs*33, VAF ~0.41; c.3261dup, p.F1088Lfs*5, VAF 0.34) <i>MSH2</i> : 1 somatic mutation (c.374CNT, p.P125L, VAF 0.42)	<i>MSH6</i> : 1 somatic mutation (c.1815_1816del, p.K606Nfs*33, VAF 0.33) <i>MSH2</i> : 1 somatic mutation (c.374CNT, p.P125L, VAF 0.32) <i>MLH1</i> : 1 somatic mutation (c.1489del, p.R497Gfs*11, VAF 0.31)
4	63	F	Splenic flexure	T4aN0	60%	P	P	P	No	<i>PMS2</i> likely pathogenic mutation (c.319CNT, p.R107W) ^c	<i>MSH6</i> : 2 somatic mutations (c.3128del, p.N1043Ifs*9, VAF 0.19; c3260_3261dup, p.F1088Pfs*3, VAF ~0.14) <i>PMS2</i> : germline mutation (c.319CNT, p.R107W); 1 somatic mutation (c.939TNA, p.Y313X, VAF 0.37);	<i>MSH6</i> : 2 somatic mutations (c.3128del, p.N1043Ifs*9, VAF 0.19; c3260_3261dup, p.F1088Pfs*3, VAF ~0.14) <i>PMS2</i> : germline mutation (c.319CNT, p.R107W); 1 somatic mutation (c.939TNA, p.Y313X, VAF 0.14);
5	71	M	Transverse	N/A	8%	P	A	A	Yes	No ^b	N/A	N/A
6	57	F	Ascending x3	T2 N0	12%	P	A	A	No	<i>MLH1</i> pathogenic mutation (exon 16-19 deletion) ^c	N/A	N/A
7	83	F	Ascending	T3 N0	15%	H	A	A	Yes	No ^b	N/A	N/A
8	77	F	Rectum	T4bM1	20%	P	P	A	No	<i>PMS2</i> pathogenic	N/A	N/A

#	Age (y)	Sex	Tumor site	pTNM	MSH6 lost area	MSH2	MLH1	PMS2	MLH1 methylation ^a	Germline mutation	MMR and POLE/POLD1 genetic alterations in tumor areas with lost MSH6 staining	MMR and POLE/POLD1 genetic alterations in tumor areas with retained MSH6 staining
9	70	M	Ascending	T2 N0	5%	P	A	A	Yes	No ^b	N/A	N/A

mutation (exon 8 deletion)^c

Abbreviations: A, absent; H, heterogeneous staining; N/A, not available; P, present.

^aMLH1 methylation was done using pyrosequencing.

^bGermline NGS of MMR genes was performed at InVita.

^cGermline NGS of MMR genes was performed at University of Washington.