Observational Study

Medicine

OPEN

Both MLH1 deficiency and *BRAF*V600E mutation are a unique characteristic of colorectal medullary carcinoma

An observational study

Masanao Kaneko, MD[a], Mitsuko Nakashima, MD, PhD[b], Kiichi Sugiura, MD[a], Natsuki Ishida, MD, PhD[c], Satoshi Tamura, MD, PhD[a], Shinya Tani, MD, PhD[a], Mihoko Yamade, MD, PhD[a], Yasushi Hamaya, MD, PhD[a], Satoshi Osawa, MD, PhD[c], Kyota Tatsuta, MD[d], Kiyotaka Kurachi, MD, PhD[d], Satoshi Baba, MD, PhD[e], Yuji Iwashita, PhD[f], Tomio Arai, MD, PhD[g], Haruhiko Sugimura, MD, PhD[f], Masato Maekawa, MD, PhD[h], Ken Sugimoto, MD, PhD[a], Moriya Iwaizumi, MD, PhD[h,*]

Abstract

Although immunohistochemistry (IHC) for mismatch repair (MMR) proteins (MMR IHC) is used to identify DNA MMR status, universal screening of all patients with colorectal cancer (CRC) using a combination of both MMR IHC and genetic testing for the *BRAF*V600E mutation is limited in Japan. This study aimed to better understand the histopathological characteristics of CRCs, which exhibit both deficient mismatch repair (dMMR) and *BRAF*V600E mutation. MMR IHC of formalin-fixed paraffin-embedded tissues from tumor areas obtained from 651 patients with CRC who underwent surgical resection at Hamamatsu University Hospital (Hamamatsu, Japan) between August 2016 and March 2022 were used to evaluate MMR status, which was determined by staining for the expression of 4 MMR proteins (MLH1, MSH2, PMS2, and MSH6). All dMMR tumors were additionally evaluated for *BRAF*V600 mutation status via Sanger sequencing. Patient clinical characteristics (age, sex, tumor location, size, and tumor pathology) were then classified using their dMMR and *BRAF*V600 mutation statuses. Among the 651 patients with CRC, 58 carried tumors with dMMR, of which 52 were deficiency in MLH1 (dMLH1). Interestingly, all 16 medullary carcinomas that were analyzed showed characteristics corresponding to the presence of both dMLH1 and *BRAF*V600E mutation ($P = .01$). These results suggest that colorectal medullary carcinomas can be diagnosed based on their unique characteristics of harboring the *BRAF*V600E mutation and exhibiting dMLH1 expression.

Abbreviations: CRC = colorectal cancer, dMLH1 = deficiency in MLH1, dMMR = deficient mismatch repair, FFPE = formalin-fixed paraffin-embedded, ICI = immune checkpoint inhibitor, IHC = immunohistochemistry, MMR = mismatch repair, MSI-H = microsatellite instability-high, pMMR = proficient MMR.

Keywords: *BRAF*, colorectal cancer, DNA mismatch repair, medullary carcinoma, MLH1

The study was supported by the Japan Society for the Promotion of Science (JSPS) (KAKENHI grant number 22K08053 to MI) and the grants from the Smoking Research Foundation (grant number 108 to HS and TA).

The present study was approved by the Ethics Committee of the Hamamatsu University School of Medicine (approval no. 2022-143). Written informed consent was obtained from all patients. The patient provided written informed consent for publication of her data and images.

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplemental Digital Content is available for this article.

[a] *First Department of Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan,* [b] *Department of Biochemistry, Hamamatsu University of School of Medicine, Hamamatsu, Japan,* [c] *Department of Endoscopic and Photodynamic Medicine, Hamamatsu University of School of Medicine, Hamamatsu, Japan,* [d] *Department of Surgery, Hamamatsu University of School of Medicine, Hamamatsu, Japan,* [e] *Department of Diagnostic Pathology, Hamamatsu University School of Medicine, Hamamatsu, Japan,* [f] *Department of Tumor Pathology, Hamamatsu University School of Medicine, Hamamatsu, Japan,* [g] *Department of Pathology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan,* [h] *Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan.*

**Correspondence: Moriya Iwaizumi, Department of Laboratory Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan (e-mail: iwaizumi@hama-med.ac.jp).*

How to cite this article: Kaneko M, Nakashima M, Sugiura K, Ishida N, Tamura S, Tani S, Yamade M, Hamaya Y, Osawa S, Tatsuta K, Kurachi K, Baba S, Iwashita Y, Arai T, Sugimura H, Maekawa M, Sugimoto K, Iwaizumi M. Both MLH1 deficiency and BRAFV600E mutation are a unique characteristic of colorectal medullary carcinoma: An observational study. Medicine 2023;102:38(e35022).

Received: 18 March 2023 / Received in final form: 5 August 2023 / Accepted: 9 August 2023

http://dx.doi.org/10.1097/MD.0000000000035022

1. Introduction

In recent years, the advent of immune checkpoint inhibitors (ICIs) has led to high therapeutic efficacy in colorectal cancer (CRC) with deficient mismatch repair (dMMR), a breakdown of the DNA mismatch repair system.[1] In 2017, the U.S. Food and Drug Administration approved the use of pembrolizumab, an ICI, as a therapeutic agent against all types of solid tumors with dMMR and/or microsatellite instability-high (MSI-H).[2] Because immunohistochemistry (IHC) for MMR (MMR IHC) proteins and/or MSI is a cost-effective CRC test that can be used for universal screening of Lynch syndrome and is a companion diagnostic aimed at personalized ICI therapy, it has been recommended for use in precision medicine by many medical institutions worldwide.[3–5]

Historically, MSI tests have been used to detect molecular manifestations in tumors with dMMR. The advent of techniques that enabled the use of monoclonal antibodies in IHC-based detection of mismatch repair (MMR) proteins (MSH2, MLH1, PMS2, and MSH6) in the 1990s resulted in MMR IHC being adopted as an alternative method for detecting MMR deficiency. This was largely due to the fact that MMR IHC, which has certain advantages over MSI testing, is a procedure routinely offered by general pathology laboratories at a cost that is approximately threefold less than that of MSI testing. In addition, another attractive feature of MMR IHC is that it helps identify mutated genes.[6–8] MSH2 dimerizes with MSH6, forming the functional complex, MutSα, while MLH1 dimerizes with PMS2, forming MutLα.[9–11] Although abnormalities of MSH2 and MLH1 proteins may result in the proteolytic degradation of their dimers and consequent loss of both the obligatory and secondary partner proteins (i.e., MSH6 and PMS2), the reverse is not true. Mutations that occur in the genes of the secondary proteins, MSH6 and PMS2, do not necessarily result in a concurrent loss of the obligatory proteins, MSH2 and MLH1, because the function of these secondary proteins may be compensated by other proteins, such as MSH3, MLH3, and PMS1.[12,13] Therefore, it is possible to estimate the primary functional loss of MMR proteins via the differences in MMR IHC expression patterns among the 4 MMR proteins.

Colorectal tumors with MSI-H and/or dMMR exhibit distinctive features, including a tendency to arise in the proximal colon, lymphocytic infiltrates, and a poorly differentiated, mucinous, or signet ring appearance; approximately 85% of colorectal tumors with dMMR are caused by acquired disruption of the DNA MMR system.[14–16] Although most sporadic dMMR tumors reportedly arise via hypermethylation of the promoter of *MLH1* and carry the *BRAF*V600E mutation,[17–22] little is known about the detailed histopathological characteristics of CRC with both deficiency in MLH1 (dMLH1) and *BRAF*V600E mutation, especially in Japan. This is because universal screening for CRC via MMR IHC is covered by insurance (October 2022) in our country. Moreover, precise histopathological findings of the tumors have not yet been reported. In addition, not all patients with CRC are eligible for *BRAF*V600 genetic testing under health insurance in Japan.

Here, we performed MMR IHC for 651 colorectal tumors, explored the clinicopathological characteristics of patients with dMMR, and examined histopathological and genetic features of tumors with dMMR based on a combined MMR IHC and *BRAF*V600 mutational analysis via Sanger sequencing. Overall, we demonstrated that patients with medullary carcinomas may carry both dMLH1 and *BRAF*V600E mutations.

2. Materials and methods

2.1. Patients

We studied 651 patients with CRC who underwent surgical resection at Hamamatsu University Hospital (Hamamatsu, Japan) between August 2016 and March 2022 (Fig. 1). Formalin-fixed paraffin-embedded (FFPE) tissues from all operated CRC patients were used to evaluate MMR status using MMR IHC. *BRAF*V600 mutation status was evaluated using Sanger sequencing analysis of tumors with dMMR. Disease

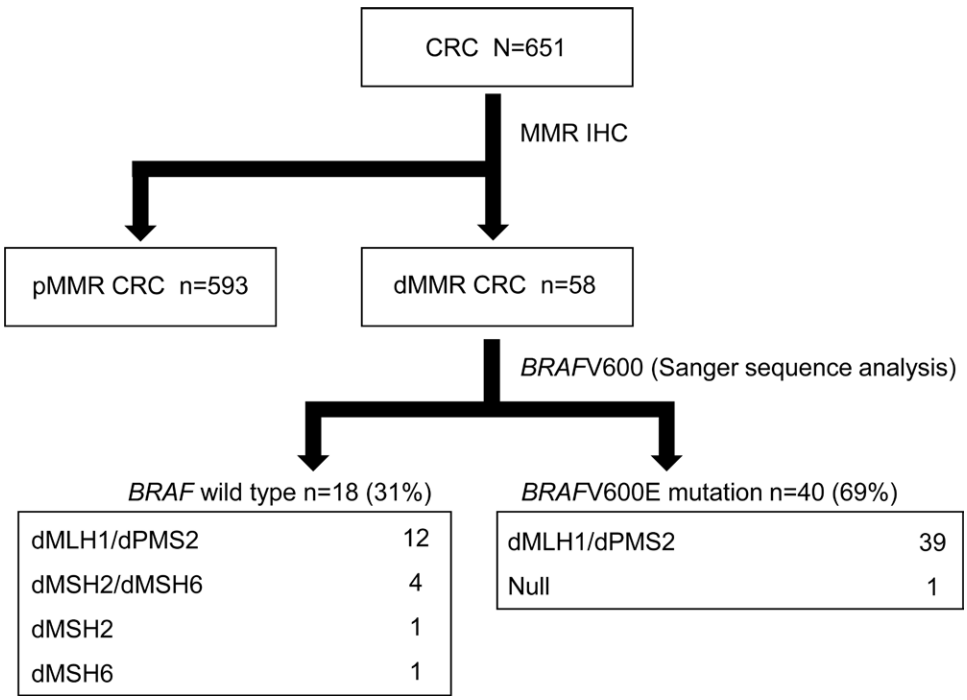

Figure 1. Tissues of patients with colorectal cancer were analyzed for DNA MMR proteins using IHC. Patients with CRC who had dMMR tumors were assessed for *BRAF* mutations using Sanger sequencing. CRC = colorectal cancer, IHC = immunohistochemistry for mismatch repair, pMMR = proficient MMR; dMMR = deficient mismatch repair; dMLH1, MHL1 deficiency; dPMS2, PMS2 deficiency; dMSH2, MSH2 deficiency; dMSH6, MSH6 deficiency; dMSH6, MSH6 deficiency; Null, tumors with no expression of all 4 MMR proteins. dMLH1 = deficiency in MLH1, MMR = mismatch repair.

status was evaluated according to the TNM staging system for CRC (Union for International Cancer Control [UICC], 8th edition). The clinical characteristics (age, sex, tumor location, tumor size, and tumor pathology) of patients were classified using MMR and *BRAF* status. Proximal colon cancer was defined as a cancer of the cecum, ascending colon, or transverse colon.

The study was approved by the Institutional Review Board of the Hamamatsu University School of Medicine (approval no. 2022-143), which confirmed that the study complied with the ethical guidelines of the Helsinki Declaration.

2.2. MMR IHC

For IHC staining, tissues were collected by experienced pathologists (H.S., T.A., and S.B.). Staining for the expression of the 4 MMR proteins, MLH1, MSH2, PMS2, and MSH6, was performed by using 10% formalin for fixation at room temperature for 6 to 48 hours. Paraffin-embedded tissue samples were cut into 4 μm thick serial sections and stained using an automated technique. Briefly, the slides were dewaxed by heating at 55°C for 30 minutes and washed thrice with xylene. Next, the tissues were rehydrated with a series of 5-minutes washes in a 100%, 95%, and 80% ethanol gradient and distilled water. Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide for 10 minutes at room temperature. Subsequently, the tissues were incubated with a protein-blocking reagent (StartingBlock [TBS] Blocking Buffer; cat. no. 37542; Thermo Fisher Scientific) at room temperature for 5 minutes, washed twice with TBS, and incubated with the mouse monoclonal antibodies anti-MLH1 (clone G168-728; 1:50; cat. 554073; BD Biosciences), anti-MSH2 (clone FE11; 1:10; cat. NA27; Merck KGaA), anti-PMS2 (clone A16–4; 1:50; cat. 556415; BD Biosciences), and anti-MSH6 (clone 44/MSH6; 1:20; cat. no. 610918, BD Biosciences) for 30 minutes at room temperature. This was followed by incubation with goat anti-mouse immunoglobulin G and horseradish peroxidase-conjugated dextran polymer (ChemMate Envision kit; Dako; Agilent Technologies, Inc.) at room temperature for 30 minutes. Antigen-antibody complexes were stained with 3,"-diaminobenzidine tetrahydrochloride using an autostainer (Histostainer; Nichirei Bioscience Corporation) and then with hematoxylin for 1 minute at room temperature. Prepared slides were observed under an optical microscope (magnification × 100 and × 400). Tissues showing positive expression, normal epithelial cells, lymphocytes, and supporting tissue were used as internal controls. The expression of MLH1, MSH2, PMS2, and MSH6 following the complete disappearance of nuclear staining in tumor cells was considered negative.

Although MLH1 is required to stabilize PMS2, PMS2 is not required to stabilize MLH1[23]; tumors lacking the expression of both MLH1 and PMS2 exhibit loss of function of MLH1 (dMLH1) and subsequent instability of PMS2. Similarly, MSH2 is required to stabilize MSH6, but MSH6 is not required to stabilize MSH2. Loss of expression of both MSH2 and MSH6 indicates loss of MSH2 function (dMSH2), followed by degradation of MSH6. Tumors that maintained the expression levels of MLH1, MSH2, PMS2, and MSH6 were assigned a proficient MMR (pMMR) status. In all cases, diagnosis was confirmed by experienced pathologists (H.S., T.A., and S.B.).

2.3. Pathological diagnosis

Experienced pathologists (H.S., T.A., and S.B.) diagnosed medullary carcinoma. According to the microscopic description of the 3rd edition of the WHO classification, medullary carcinoma is characterized by a sheet-like structure of malignant cells with vesicular nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm with prominent intraepithelial lymphocytic infiltration.[24]

2.4. Samples

Postoperative FFPE tissues obtained from patients with CRC were used as tumor samples. Genomic DNA was extracted from cancer tissue using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany).

2.5. BRAFV600 genetic testing

BRAF mutations in exon 15 were analyzed. Exon 15 was selected because it is the region where *BRAF* mutations are most frequently reported. We performed PCR using the primer set described previously (see Table, Supplemental Digital Content 1, http://links.lww.com/MD/J715 showing the primers used for PCR amplification). PCR amplifications were performed under the following conditions: 1 cycle at 94°C for 2 minutes; 40 cycles at 98°C for 10 seconds; 60°C for 30 seconds; extension at 70°C for 30 seconds; and a final extension step at 70°C. PCR products were purified using an Ampure Xp beads kit (Beckman Coulter, Blair, NE) and sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, MA) according to the manufacturer instructions. Sequencing was performed in both directions using forward and reverse PCR primers. The purified products were run on an ABI 3100 PRISM Genetic Analyzer (Applied Biosystems). Data were collected and analyzed using Applied Biosystems sequence analysis software.

2.6. Statistical analyses

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria). It is a modified version of R Commander, which was designed to add statistical functions used in biostatistics more precisely.[25]

3. Results

3.1. Colorectal medullary carcinomas exhibit dMMR

We compared the clinicopathological features of 58 patients with dMMR CRC (8.9%) and 593 patients with pMMR CRC (91.1%) (Table 1). When presenting for the study, the CRC patients in the dMMR CRC group were older than those in the pMMR CRC group. The male:female proportion was almost equal between the 2 groups. Tumor size in dMMR CRC was larger than that in pMMR CRC. The dMMR tumors were mainly located in the right-sided colon, whereas pMMR tumors were located in the left-sided colon ($P < .0001$). UICC pStage IV was more common in patients with pMMR tumors, whereas UICC pStage II was more common in patients with dMMR tumors ($P = .0002$). In terms of pathology, the dMMR CRC tumors displayed a higher proportion of medullary and mucinous carcinomas, whereas the pMMR CRCs tumors had a higher proportion of tubular adenocarcinoma. Notably, all medullary carcinomas exhibited dMMR.

3.2. All colorectal medullary carcinomas exhibited defective MLH1 expression

Because tumors with dMLH1 were the most frequent type of tumor displaying disrupted DNA MMR, we compared clinicopathological characteristics corresponding to the MLH1 expression status of dMMR tumors. The results indicated that patients with dMLH1 tumors were older than those with preserved MLH1 function ($P = .02$) and that 77% of tumors with dMLH1 carried *BRAF*V600 mutations, whereas patients with pMLH1 tumors did not ($P = .00046$) (Table 2). Interestingly, MMR IHC indicated that none of the 16 medullary carcinomas

expressed both MLH1 and PMS2 (Table 3), including 1 case that did not express any MMR protein at all (Table 4; see Figure, Supplemental Digital Content 2, http://links.lww.com/MD/J716 illustrating a case of a patient with medullary carcinoma).[26] The loss of expression of both MLH1 and PMS2 is caused by disruption of the MLH1 protein because abnormalities in the MLH1 protein result in proteolytic degradation of the MLH1/PMS2 dimer and consequent loss of both the obligatory and secondary partner protein PMS2. These results suggested that medullary carcinomas were deficient in MLH1 expression.

3.3. Colorectal medullary carcinomas displayed both MLH1 deficiency and BRAFV600 mutation

Most CRC tumors with hypermethylation of *MLH1* reportedly show the *BRAF*V600 mutation[17–22]; therefore, we analyzed the histopathological characteristics of CRC with both *BRAF*V600 mutation and deficient MLH1 protein expression. The CRC dMMR content in the MMR plot (MLH1, MSH2, PMS2, and MSH6) combined with *BRAF*V600 and tumor histology is shown (Fig. 2). Among dMMR CRCs, some tubular adenomas displayed dMLH1, while others displayed non-dMLH1 characteristics; not all mucinous carcinomas displayed dMLH1. By

Table 1

Clinicopathological comparison of the dMMR and pMMR groups in all colorectal cancers (CRCs).

	dMMR n = 58	pMMR n = 593	*P* value
Age at surgery, yr, median (range)	73.0 (43–93)	68.2 (32–95)	.004
<70	18	287	.01
≥70	40	306	
Male/female, n	28/30	345/248	.2
Tumor size, (mm), (SD)	49.0 (23.4)	41.9 (20.2)	.01
Tumor location, n (%)			<.0001
Right colon	52 (90)	195 (33)	
Left colon	3 (5)	162 (27)	
Rectum	3 (5)	236 (40)	
Tumor histology, n (%)			<.0001
Medullary carcinoma	16 (28)	0 (0)	<.0001
Poorly differentiated adenocarcinoma	1 (2)	11 (2)	1
Mucinous carcinoma	9 (15)	29 (5)	.004
Tubular or papillary adenocarcinoma	32 (55)	553 (93)	<.0001
Pathological stage, n (%)			.0002
I	15 (26)	155 (26)	1
II	28 (48)	154 (26)	.0006
III	15 (26)	214 (36)	.2
IV	0 (0)	70 (12)	.002

dMMR = deficient mismatch repair, pMMR = proficient MMR.

Table 2

Clinicopathological comparison of dMLH1 and pMLH1 in dMMR colorectal cancers (CRCs).

	dMMR (n = 58)		
	dMLH1 n = 52	pMLH1 n = 6	*P* value
Age at surgery, yr, median (range)	74.3 (43–93)	62.5 (44–84)	.02
Male/female, n	25/27	3/3	1
Tumor size, (mm), (SD)	48.5 (23.2)	50.8 (27.1)	.8
Tumor location, n (%)			1
Right colon	46 (88)	6 (100)	
Left colon	3 (6)	0 (0)	
Rectum	3 (6)	0 (0)	
Tumor histology, n (%)			.3
Medullary carcinoma	16 (31)	0 (0)	
Poorly differentiated adenocarcinoma	1 (2)	0 (0)	
Mucinous carcinoma	8 (15)	1 (17)	
Tubular or papillary adenocarcinoma	27 (52)	5 (83)	
Pathological stage, n (%)			.3
I	15 (28)	0 (0)	
II	25 (48)	3 (50)	
III	12 (23)	3 (50)	
IV	0 (0)	0 (0)	

dMLH1 = deficiency in MLH1, dMMR = deficient mismatch repair, pMLH1 = MLH1 proficiency, SD = standard deviation.

Table 3

Clinicopathological features of all medullary carcinoma cases.

	Medullary carcinoma n = 16
Age at surgery, yr, median (range)	75.4 (43–88)
Male/female, n	7/9
Tumor size, (mm), (SD)	60.8 (22.3)
Tumor location, n (%)	
Right colon	15 (94)
Left colon	0 (0)
Rectum	1 (6)
Pathological stage, n (%)	
I	2 (13)
II	8 (50)
III	6 (37)
IV	0 (0)

SD = standard deviation.

Table 4

List of clinicopathological features of all medullary carcinoma cases.

Case no.	Age	Sex	Tumor location	Tumor size (mm)	UICC pStage	MMRP
1	88	F	A	110	IIIC	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
2	81	M	A	70	IIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
3	43	M	A	50	IIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
4	73	F	A	48	IIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
5	83	F	A	90	IIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
6	85	F	A	47	IIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
7	66	F	A	85	IIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
8	77	M	A	60	IIB	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
9	71	M	A	80	IIC	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
10	81	M	A	37	I	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
11	79	F	A	25	I	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
12	72	M	C	40	IIIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
13	76	F	T	60	IIIC	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
14	82	F	T	70	IIIC	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
15	59	F	R	45	IIIA	MLH1(–), PMS2(–), MSH2(+), MSH6(+)
16	70	M	A	55	IIIB	MLH1(–), PMS2(–), MSH2(–), MSH6(–)

A = ascending colon, C = cecum, MMRP = mismatch repair protein, MT = mutation, R = rectum, T = transverse colon

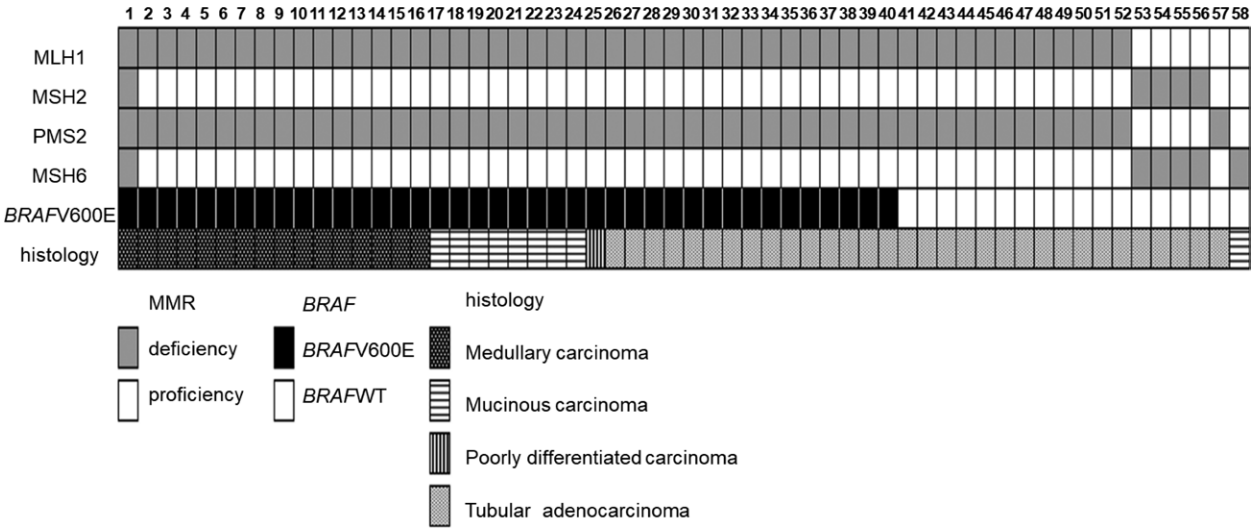

Figure 2. MMR status and *BRAF* mutation status in dMMR CRC, including histology. The upper 4 blocks show the representation of MMR proteins: gray if deficient; white if proficient. Fifth block from the top indicates whether tumors have *BRAF*V600E mutations: presence of a mutation is indicated by black and absence is indicated by white. Bottom block shows histopathological diagnosis, leading to classification as medullary carcinoma, mucinous carcinoma, tubular adenocarcinoma, and poorly differentiated adenocarcinoma. CRC = colorectal cancer, dMMR = deficient mismatch repair, MMR = mismatch repair.

Table 5

Differences between medullary and non-medullary carcinomas according to *BRAF*V600E status in dMLH1 colorectal cancers (CRCs).

		dMLH1 (n = 52)		
		***BRAF*V600E (n = 40)**	***BRAF*WT (n = 12)**	***P* value**
Medullary carcinoma (%)		16 (40.0)	0 (0.0)	.01
Non medullary carcinoma	Poorly differentiated adenocarcinoma (%)	1 (2.5)	0 (0.0)	
	Mucinous carcinoma (%)	8 (20.0)	0 (0.0)	
	Tubular adenocarcinoma (%)	15 (37.5)	12 (30.0)	

dMLH1 = deficiency in MLH1, WT = wild-type.

contrast, all the medullary carcinomas and 1 poor adenocarcinoma, among those with dMMRs, displayed only dMLH1 (Table 5), while some poor adenocarcinomas exhibited pMMR (Table 1). These results indicated that dMLH1 is a unique characteristic of medullary carcinomas. Our focus on *BRAF*V600 mutation status among dMLH1 CRCs led to an insightful finding that all the medullary carcinomas that were analyzed carried the *BRAF*V600 mutation, whereas some tumors among the non-medullary carcinomas displayed pMLH1 and/or *BRAF*V600 WT. Thus, these results suggested that medullary carcinoma of the large intestine displays the unique characteristics of both dMLH1 and *BRAF*V600 mutations.

4. Discussion

Medullary carcinoma was first described in the 3rd edition of the WHO classification in 2000.[24,27,28] The pathologic features of colorectal medullary carcinoma include sheets of malignant cells with vesicular nuclei, prominent nucleoli, medullary growth with poor lumen formation, relatively abundant eosinophilic cytoplasm, and diffuse infiltration of T lymphocytes called tumor-infiltrating lymphocytes.[29] Recently, Jabbal et al utilized the National Cancer Database from 2004 to 2018 to show that medullary carcinoma is prevalent among older patients and women and that 82.4% of patients (mostly including patients with sporadic CRCs) with medullary carcinoma show MSI.[30] Although CRCs with MSI-H exhibited hypermethylation of *MLH1*, and most *MLH1* hypermethylated tumors carried the *BRAF*V600 mutation,[16] reports pertaining to the most typical histological type among CRCs, which carries both mutated *BRAF*V600 and disrupted MLH1, are scarce. To the best of our knowledge, our study is the first report to show that colorectal medullary carcinomas display characteristics of both deficient MLH1 (via MMR IHC) and mutated *BRAF*V600 (via Sanger sequencing).

Most sporadic CRCs with dMMR may be characterized by *MLH1* promoter hypermethylation,[14–16] and many studies have reported that colorectal tumors showing *MLH1* promoter hypermethylation exhibit the histopathological characteristic of mucinous or poor differentiation. The absence of studies discussing medullary carcinoma in detail may be attributable to these analyses being conducted prior to or just after the definition of medullary carcinoma was released in the year 2000 by WHO, suggesting that medullary carcinoma may be categorized as poorly differentiated adenocarcinoma.[17–22] In 2004, Arai et al studied 35 poorly differentiated adenocarcinoma cases, including 23 medullary carcinomas in the elderly. They found that of the 23 medullary carcinomas, 20 carried MSI-H, 21 carried dMLH1, and 15 carried *MLH1* promoter hypermethylation.[31] Although the results of their study are insightful, they did not show the frequency of colorectal medullary carcinoma among all patients with surgically resected CRC as well as the frequency of medullary carcinomas with dMLH1, which has rarely been reported. By contrast, our study makes a strong case by showing that among 651 resected CRCs, all 16 medullary carcinomas that were analyzed displayed dMLH1, whereas none showed pMLH1.

The CpG island methylator phenotype, which occurs in ~20% of sporadic CRCs, mostly demonstrates *BRAF*V600 mutation and *MLH1* hypermethylation, and thus represents hypermutated tumors.[32–35] This has also been experimentally demonstrated using mouse models in which induction of the *BRAF* mutation results in consistent DNA methylation changes that are analogous to CpG island methylator phenotype in human CRC.[36,37] Focusing on medullary carcinoma, Knox et al[38] analyzed a single health district database from 1998 to 2012 and showed that 85.6% of medullary carcinomas carried *BRAF*V600E mutations, while 100% carried dMMR. Although these findings may be important, they do not show *BRAF*V600E mutation status together with MLH1 expression. Our study had some

limitations: this was a single-center retrospective study and we did not analyze unresected advanced CRC cases. However, our findings have successfully and clearly demonstrated that medullary colorectal carcinomas display the characteristics of both MLH1 deficiency and *BRAF*V600E mutation. Considering the difficulties associated with distinguishing between medullary carcinoma with neuroendocrine tumors and poorly differentiated adenocarcinoma, we believe that universal screening for both dMLH1 and *BRAF*V600E mutations as unique biomarkers may help diagnose medullary carcinoma to decide on the course of treatment.

Author contributions

Conceptualization: Moriya Iwaizumi.
Data curation: Masanao Kaneko, Mitsuko Nakashima, Satoshi Osawa, Kiyotaka Kurachi, Moriya Iwaizumi.
Formal analysis: Masanao Kaneko, Natsuki Ishida, Kyota Tatsuta, Satoshi Baba, Tomio Arai, Haruhiko Sugimura, Masato Maekawa, Ken Sugimoto, Moriya Iwaizumi.
Funding acquisition: Moriya Iwaizumi.
Investigation: Masanao Kaneko, Kiichi Sugiura, Natsuki Ishida, Satoshi Tamura, Yasushi Hamaya, Satoshi Baba, Yuji Iwashita, Tomio Arai, Moriya Iwaizumi.
Methodology: Moriya Iwaizumi.
Project administration: Masanao Kaneko.
Validation: Moriya Iwaizumi.
Visualization: Moriya Iwaizumi.
Writing – original draft: Masanao Kaneko, Mitsuko Nakashima, Natsuki Ishida, Satoshi Tamura, Shinya Tani, Yasushi Hamaya, Kyota Tatsuta, Kiyotaka Kurachi, Tomio Arai, Haruhiko Sugimura, Masato Maekawa, Ken Sugimoto, Moriya Iwaizumi.
Writing – review & editing: Mitsuko Nakashima, Satoshi Tamura, Shinya Tani, Mihoko Yamade, Yasushi Hamaya, Satoshi Osawa, Kyota Tatsuta, Kiyotaka Kurachi, Satoshi Baba, Yuji Iwashita, Tomio Arai, Haruhiko Sugimura, Masato Maekawa, Ken Sugimoto, Moriya Iwaizumi.

References

[1] Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015;372:2509–20.
[2] Marcus L, Lemery SJ, Keegan P, et al. FDA approval summary: pembrolizumab for the treatment of microsatellite instability-high solid tumor. Clin Cancer Res. 2019;25:3753–8.
[3] Mvundura M, Grosse SD, Hampel H, et al. The cost-effectiveness of genetic testing strategies for Lynch syndrome among newly diagnosed patients with colorectal cancer. Genet Med. 2010;12:93–104.
[4] Tomita N, Ishida H, Tanakaya K, et al. Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2020 for the Clinical Practice of Hereditary Colorectal Cancer. Int J Clin Oncol. 2021;26:1353–419.
[5] Cutsem EV, Cervantes A, Adam R, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol. 2016;27:1386–422.
[6] Wilson TM, Ewel A, Duguid JR, et al. Differential cellular expression of the human MSH2 repair enzyme in small and large intestine. Cancer Res. 1995;55:5146–50.
[7] Leach FS, Polyak K, Burrell M, et al. Expression of the human mismatch repair gene hMSH2 in normal and neoplastic tissues. Cancer Res. 1996;56:235–40.
[8] Debniak T, Kurzawski G, Gorski B, et al. Value of pedigree/clinical data, immunohistochemistry and microsatellite instability analyses in reducing the cost of determining hMLH1 and hMSH2 gene mutations in patients with colorectal cancer. Eur J Cancer. 2000;36:49–54.
[9] Acharya S, Wilson T, Gradia S, et al. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci USA. 1996;93:13629–34.
[10] Kadyrov FA, Dzantiev L, Constantin N, et al. Endonucleolytic function of MutLalpha in human mismatch repair. Cell. 2006;126:297–308.
[11] Harfe BD, Minesinger BK, Jinks-Robertson S. Discrete in vivo roles for the MutL homologs Mlh2p and Mlh3p in the removal of frameshift intermediates in budding yeast. Curr Biol. 2000;10:145–8.
[12] Chang DK, Ricciardiello L, Goel L, et al. Steady-state regulation of the human DNA mismatch repair system. J Biol Chem. 2000;275:29178.
[13] Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. J Mol Diagn. 2008;10:293–300.
[14] Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA. 1998;95:6870–5.
[15] Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med. 2003;348:919–32.
[16] Boland CR, Goel A. Microsatellite instability in colorectal cancer. Gastroenterology. 2010;138:2073–2087.e3.
[17] Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res. 1997;57:808–11.
[18] Koinuma K, Shitoh K, Miyakura Y, et al. Mutations of BRAF are associated with extensive hMLH1 promoter methylation in sporadic colorectal carcinomas. Int J Cancer. 2004;108:237–42.
[19] Cunningham JM, Christensen ER, Tester DJ, et al. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res. 1998;58:3455–60.
[20] Veigl ML, Kasturi L, Olechnowicz J, et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci USA. 1998;95:8698–702.
[21] Young J, Simms LA, Biden KG, et al. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol. 2001;159:2107–16.
[22] Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology. 2002;122:1376–87.
[23] Fleming M, Ravula S, Tatishchev SF, et al. Colorectal carcinoma: pathologic aspects. J Gastrointest Oncol. 2012;3:153–73.
[24] Hamilton SR, Aaltonen LA. WHO Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System. Lyon: IARC; 2000.
[25] Kanda Y. Investigation of the freely available easy-to-use software "EZR" for medical statistics. Bone Marrow Transplant. 2013;48:452–8.
[26] Tatsuta K, Sakata M, Iwaizumi M, et al. Mismatch repair proteins immunohistochemical null phenotype in colon medullary carcinoma. Clin J Gastroenterol. 2021;14:1448–52.
[27] Jessurun J, Romero-Guadarrama M, Manivel JC. Medullary adenocarcinoma of the colon: clinicopathologic study of 11 cases. Hum Pathol. 1999;30:843–8.
[28] Thirunavukarasu P, Sathaiah M, Singla S, et al. Medullary carcinoma of the large intestine: a population based analysis. Int J Oncol. 2010;37:901–7.
[29] Pyo JS, Sohn JH, Kang G. Medullary carcinoma in the colorectum: a systematic review and meta-analysis. Hum Pathol. 2016;53:91–6.
[30] Jabbal IS, Nagarajan A, Rivera C, et al. Medullary carcinoma of the colon: a comprehensive analysis of the National Cancer Database. Surg Oncol. 2022;45:101856.
[31] Arai T, Esaki Y, Sawabe M, et al. Hypermethylation of the hMLH1 promoter with absent hMLH1 expression in medullary-type poorly differentiated colorectal adenocarcinoma in the elderly. Mod Pathol. 2004;17:172–9.
[32] Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology. 2008;135:1079–99.
[33] Carethers JM, Jung BH. Genetics and genetic biomarkers in sporadic colorectal cancer. Gastroenterology. 2015;149:1177–1190.e3.
[34] Willett CG, Chang DT, Czito BG, et al. Cancer Genome Atlas Network Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487:330–7.
[35] Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 2006;38:787–93.
[36] Bond CE, Liu C, Kawamata F, et al. Oncogenic BRAF mutation induces DNA methylation changes in a murine model for human serrated colorectal neoplasia. Epigenetics. 2018;13:40–8.
[37] Fennell L, Kane A, Liu C, et al. BRAF mutation induces rapid neoplastic transformation in the aged and aberrantly methylated intestinal epithelium. Gut. 2022;71:1127–40.
[38] Knox RD, Luey N, Sioson L, et al. Medullary colorectal carcinoma revisited: a clinical and pathological study of 102 cases. Ann Surg Oncol. 2015;22:2988–96.