



Next-generation sequencing reveals heterogeneous genetic alterations in key signaling pathways of mismatch repair deficient colorectal carcinomas

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Abstract

Colorectal carcinoma (CRC) with deficient mismatch repair (dMMR) is an etiologically heterogeneous molecular entity. We investigated the genetic profile, focusing on key signaling pathways and molecular diversity of dMMR CRCs. In this study, next-generation sequencing was applied to 156 consecutive dMMR CRCs and 225 randomly selected proficient MMR (pMMR) CRCs diagnosed between July 2015 and December 2019 at Peking Union Medical College Hospital. Genetic alterations and *MLH1* promoter hypermethylation (*MLH1*^{me+}) were analyzed. Among the most frequently mutated genes, *RNF43*, *ARID1A*, *PIK3CA*, *ATM*, and *BRCA2* mutants were enriched in dMMR CRCs, whereas *APC* and *TP53* mutations were enriched in pMMR CRCs. In dMMR group, *RNF43*, *APC*, *ARID1A*, and *BRCA2* mutations were largely microsatellite instability events. WNT pathway was commonly altered regardless of MMR status. Compared to pMMR CRCs, dMMR CRCs had remarkably more prevalent PI3K, RTK-RAS, TGFβ, and DNA damage repair pathway alterations and more multiple mutations in WNT and PI3K pathways. Within dMMR tumors, mutual exclusivity occurred between *CTNNB1* mutation and *APC* or *RNF43* mutation, while coexistence existed between *BRAF* and *RNF43* mutation, as well as *RAS* and *APC* mutation. *MLH1*^{me+} dMMR CRCs had significantly more frequent *RNF43* mutations but less frequent *KRAS*, *APC*, and *CTNNB1* mutations comparing to *MLH1*-unmethylated dMMR CRCs. *RNF43/BRAF* comutations were detected in *MLH1*^{me+} dMMR CRCs, whereas *RAS/APC* comutations were largely detected in Lynch syndrome-associated cases. *RNF43* mutation was independently associated with *MLH1*^{me+} rather than *BRAF* mutations. dMMR CRCs bearing receptor tyrosine kinase fusion demonstrated no additional RTK-RAS mutations, significantly fewer PI3K alterations and more *TGFBR2* mutations than other dMMR tumors. Our study revealed that dMMR CRCs had distinctive gene mutation spectra and signaling pathway interaction patterns compared to proficient mismatch repair (pMMR) CRCs, and molecular heterogeneity was evident for these divergent oncogenic pathways. These findings justify the use of individualized therapy targeted to dMMR CRC subgroups.

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Introduction

Colorectal carcinoma (CRC) is one of the most prevalent malignancies worldwide, ranking as the third and fifth leading cause of cancer-related death in the USA and China [1, 2], respectively. Currently, although AJCC-TNM staging remains the main basis of treatment decision making, it

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has increasingly been shown to be insufficient for accurate prognosis or reliable personalized therapy.

Recent advancements in high-throughput genomic studies have revealed divergent intrinsic molecular features of CRC, which are of critical prognostic and predictive significance. One of these is deficient DNA mismatch repair (dMMR), leading to a phenotype of high-level microsatellite instability (MSI-H) in ~15% of CRCs [3]. Clinical studies have indicated a more favorable prognosis for patients with stage II/III dMMR CRCs. Most notably, dMMR status is associated with increased tumor mutational and neoantigen burdens, higher density of tumor-infiltrating lymphocytes and immune checkpoint protein compensatory upregulation and has been highlighted as an indication for the application of checkpoint blockade therapy in multiple solid tumors, including CRC [4–6]. However, a comprehensive characterization of the cancer-driving genes and signaling pathways that distinguish dMMR has not been thoroughly illustrated. In particular, MMR has been established as a crucial part of the DNA damage response (DDR) system, and the process is composed of a series of distinct yet functionally interwoven pathways that maintain genomic integrity [7]. The complexity and clinical relevance of the interaction between DDR pathways has been elucidated in lung adenocarcinoma [8] and is worthy of further exploration in dMMR CRCs.

Traditionally, three main etiologically distinct subgroups have been identified in dMMR CRCs [9]. The most common one is “sporadic dMMR CRCs,” of which the dMMR status is primarily attributed to epigenetic hypermethylation of the *MLH1* promoter region and subsequent transcriptional silencing. Another subgroup consists of “Lynch syndrome-associated dMMR CRCs,” carrying deleterious germline alterations in MMR genes *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM*. Tumors bearing neither *MLH1* hypermethylation nor germline MMR-mutated genes comprise a group of “unexplained dMMR CRCs” and are associated with a variety of possible causes. This molecular categorization has been emphasized for its significance in genetic risk evaluation and management. Nevertheless, the underlying molecular heterogeneity and implications for targeted therapy remain to be clarified. Furthermore, we and others [10–13] have recently recognized a distinguishable molecular subgroup composed of CRC tumors harboring therapeutically targetable receptor tyrosine kinase (RTK) fusion proteins, which are extremely uncommon but enriched in dMMR CRCs with *MLH1* hypermethylation. Although certain molecular features of this subgroup have been previously described, an in-depth understanding awaits thorough study and comparison with other dMMR subgroups.

In the present study, we investigated the detailed molecular and genetic features of a consecutive dMMR CRC

cohort, including subgroups, by comprehensive genomic profiling (CGP), and the findings are then compared with features of proficient MMR (pMMR) CRCs. We focused on mutations in key genes affecting the WNT, RTK-RAS, PI3K, TGF- β , TP53, and DDR pathways. The mutual relationships of alterations within the same and among different pathways were also explored. Herein, we demonstrate that dMMR CRCs display a characteristic yet remarkably heterozygous gene mutation profile and pathway interaction pattern, which might have important implications for individualized targeted therapy.

Materials and methods

Patient selection

The materials used in this retrospective study were collected from pathology archives of Peking Union Medical College Hospital (PUMCH) and included data of patients diagnosed with CRC between July 2015 and December 2019. All patients with data included in the study underwent partial colectomy for primary CRC. No patients were known to have received neoadjuvant therapy or tyrosine kinase inhibitor therapy. Tumors with intact immunohistochemical (IHC) staining of all four MMR proteins of interest [14] were identified as dMMR tumors. The dMMR group ($n = 156$) comprised consecutive cases, whereas the pMMR group ($n = 225$) comprised randomly selected cases. This study was approved upon ceding review by the PUMCH Institutional Review Board for review.

Comprehensive genomic profiling (CGP)

CGP was performed using hybrid capture-based targeted next-generation sequencing as previously described [15, 16]. In brief, DNA from formalin-fixed paraffin-embedded colorectal tumors and patient-matched normal tissues was extracted and sheared. Barcoded libraries were hybridized to our customized panel of 1021 genes containing whole exons, selected introns of 288 genes, and selected regions of 733 genes (Supplementary Table 1). The libraries were prepared and sequenced to a uniform median depth ($>500\times$). Genomic alterations, including single nucleotide variants, small insertions and deletions, copy number alterations, and gene fusions/rearrangements, were compared against each patient’s corresponding normal sample. MSI status was determined using MSIsensor (v0.2), which reported the percentage of unstable somatic microsatellites through a Chi-square test on predefined microsatellite regions covered by our panel. The loss of heterozygosity (LOH) in MMR genes was determined by an

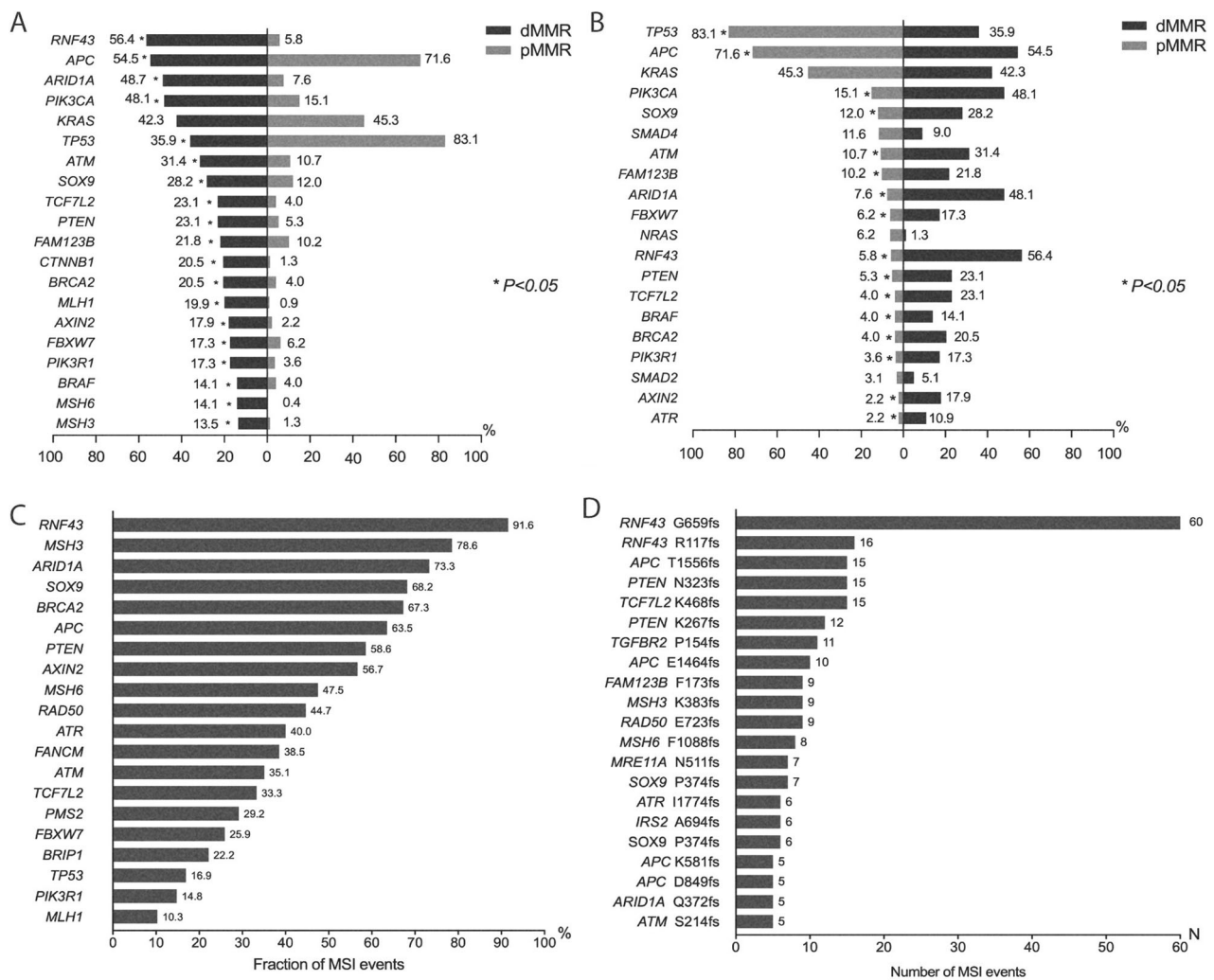


Fig. 1 Mutation profile of recurrently mutated genes in the dMMR and pMMR CRCs. **a** Prevalence of most frequently mutated genes in the dMMR CRCs compared with that in the pMMR CRCs. **b** Prevalence of the most frequently mutated genes in the pMMR CRCs compared with that in the dMMR CRCs. **c** Fraction of MSI-related somatic mutations of the 20 most recurrently mutated tumor

suppressor genes in the dMMR CRCs. **d** Bar chart showing the most commonly detected MSI-related genes in the dMMR CRCs. dMMR deficient mismatch repair, pMMR proficient mismatch repair, CRC colorectal carcinoma, MSI microsatellite instability. Asterisk (*) significant difference in mutation prevalence (Chi-square test or Fisher's exact test, $P < 0.05$).

analysis of the variant allele fraction (VAF) using a local algorithm modified based on a previously reported method [14]. LOH was established when (1) the VAF for a mutation was $>80\%$ higher than the average VAF for somatic mutations in the tumor and when (2) LOH was verified by shifts in the expected VAFs, which indicate germline polymorphisms within the same gene region. A possible LOH was identified when the VAF of a mutation was between 40 and 80% higher than the average VAF for somatic mutations in the tumor. The average sequencing depth for the target regions of the tumor samples was 1026x, and 99.0% of the average coverage of the targeted regions was more than 200x, which were qualified for variant calling and the MSI analysis.

Key genes and determination of mutational significance

Within our 1021-gene panel, 26 genes involved in the WNT, RTK-RAS, PI3K, TGFβ, and TP53 pathways were recognized as genes with recurring mutations in key CRC signaling pathways, as determined by TCGA analysis [17]. Fifty-six genes has been identified as DDR-related genes in previous studies [18–20] and were assigned to eight DDR pathways: MMR, homologous recombination repair (HRR), Fanconi anemia (FA), base excision repair, checkpoint factor, nucleotide excision repair, nonhomologous end joining, and translesion DNA synthesis (Supplementary Table 2).

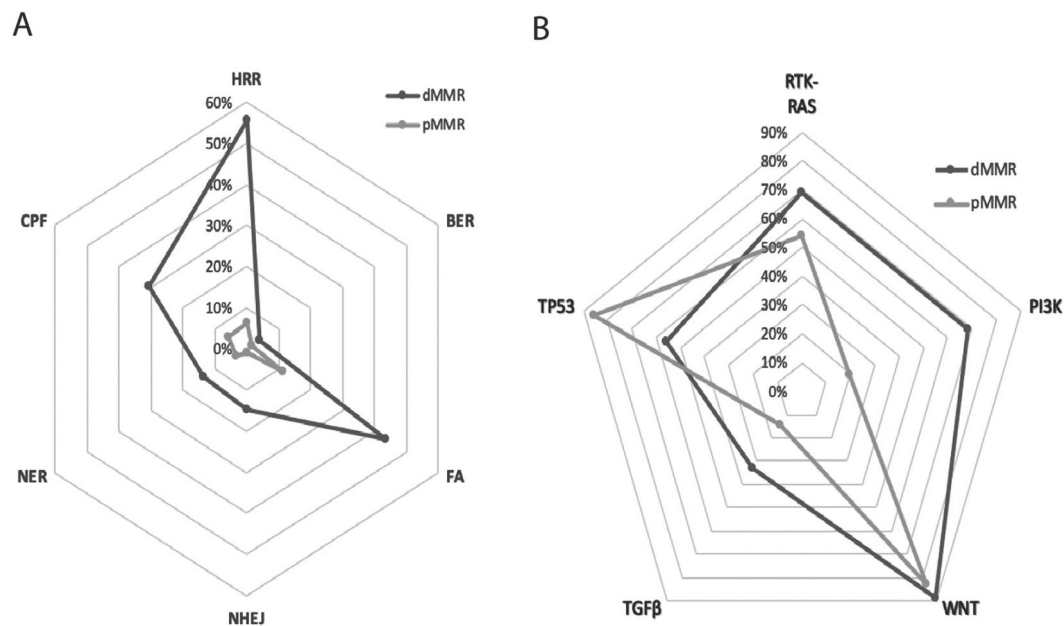


Fig. 2 Distribution of DNA damage repair pathways, and WNT, RTK-RAS, PI3K, TGF β and TP53 pathways with respect to their mutation frequency in the dMMR and pMMR CRCs. (a) Distribution of DNA damage repair pathways (apart from mismatch repair pathway); (b) Distribution of WNT, RTK-RAS, PI3K, TGF β and

TP53 pathways. dMMR deficient mismatch repair, pMMR proficient mismatch repair, CRC colorectal carcinoma, HRR homologous recombination repair, FA Fanconi anemia, BER base excision repair, CPFs checkpoint factors, NER nucleotide excision repair, NHEJ nonhomologous end joining, and TLS translesion synthesis.

Mutations of oncogenes were filtered according to the related documentation in the Catalog of Somatic Mutations in Cancer (COSMIC) [21] and OncoKB annotation [22]. For tumor suppressor genes, only “predicted deleterious” mutations were included in our analysis. All loss-of-function mutations, including nonsense mutations, frame-shift mutations, and mutations affecting canonical splice sites were defined as “predicted deleterious” mutations. Missense mutations were considered deleterious when identified in two or more of the following *in silico* functional analysis algorithms: prediction score 0.0–0.05 in SIFT (sorting intolerant from tolerant) [23], “possibly damaging” or “probably damaging” in polymorphism phenotyping-2 (Polyphen2) [24], or “medium” or “high” in MutationAssessor [25]. For missense mutations in the DDR pathway, the functional effects were manually reviewed in the COSMIC, recurrent hotspot mutations [26] and PubMed searches, as previously described by Iyer et al. [27] and Teo et al. [28].

MLH1 promoter hypermethylation analysis

All tumors that presented with the loss of MLH1 expression and the lack of MMR germline mutations were analyzed for *MLH1* promoter hypermethylation using methylation-specific PCR, with the protocol detailed in our previous report [10].

Statistical methods

Categorical variables were expressed as percentages. Chi-square test or Fisher’s exact test was used when appropriate to compare the frequencies of genetic alterations and to identify the coexistence or mutually exclusive associations. Correlation analysis was conducted using binary logistic regression. Statistical processing was performed using SPSS version 23 (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ (two-sided) was considered significant.

Results

Gene mutations in the dMMR and pMMR CRCs

Of the key genes involved in WNT, RTK-RAS, PI3K, TGF β , TP53, and DDR pathways, we found that *RNF43* (56.4%), *APC* (54.5%), *ARID1A* (48.7%), *PIK3CA* (48.1%), *KRAS* (42.3%), *TP53* (35.9%), *ATM* (31.4%), *SOX9* (28.2%), *TCF7L2* (23.1%), *PTEN* (23.1%), *FAM123B* (21.8%), *CTNNB1* (20.5%), *BRCA2* (20.5%), *MLH1* (19.9%), and *AXIN2* (17.9%) were the most frequently somatic genes mutated in the dMMR CRCs analyzed (Fig. 1a, Supplementary Table 3), whereas *TP53* (83.1%), *APC* (71.6%), *KRAS* (45.3%), *PIK3CA* (15.1%), *SOX9* (12.0%), *SMAD4* (11.6%), *ATM* (10.7%), *ARID1A*

(7.6%), *FBXW7* (6.2%), *NRAS* (6.2%), *RNF43* (5.8%), *PTEN* (5.3%), *TCF7L2* (4.0%), and *BRAF* (4.0%) were the genes most frequently mutated in the pMMR CRCs analyzed (Fig. 1b, Supplementary Table 3). In comparison with those in the pMMR CRCs, the mutation rates of the majority of recurrently mutated genes in our analysis were significantly higher in the dMMR group ($P < 0.05$), while the *APC* and *TP53* mutations were the only genomic alterations significantly enriched in the pMMR CRCs (54.5% vs. 71.6%, $P = 0.001$; 33.9% vs. 83.1%, $P < 0.001$). The prevalence of mutations in *KRAS*, *NRAS*, *SMAD2*, *SMAD4*, and *RAD51* did not differ significantly between the dMMR and pMMR groups.

Of the recurrently mutated tumor suppressor genes in the dMMR CRCs, the majority of alterations in *RNF43* (87/95, 91.6%), *MSH3* (22/28, 78.6%), *ARID1A* (55/75, 73.3%), *SOX9* (30/44, 68.2%), *BRCA2* (33/49, 67.3%), *APC* (54/85, 63.5%), *PTEN* (34/58, 58.6%), and *AXIN2* (17/30, 56.7%) were found to be insertions or deletions affecting homopolymer sequences and were recognized as MSI events (Fig. 1c). Specifically, the most commonly detected MSI-related alterations in the dMMR CRCs were the well-known *RNF43* variants: p. G659fs and p. R117fs (Fig. 1d). Somatic mutations affecting *PMS2*, *FBXW7*, *BRIP1*, *TP53*, *PIK3R1*, and *MLH1* were largely nonsense mutations or missense mutations predicted to be deleterious.

Signaling pathway alterations in the dMMR and pMMR CRCs

DDR-related pathway deficiencies were found to be exceedingly common among the dMMR CRCs, in contrast to the low frequencies in the pMMR CRCs. In addition to the MMR pathway, which was damaged in all the dMMR cases, the HRR and FA pathways were the most commonly mutated DDR pathways, affecting 55.8% (87/156) and 43.6% (68/156) of the dMMR tumors, respectively (Fig. 2a).

Among defects in other key pathways driving CRC carcinogenesis, defects in the WNT signaling pathway were common to both groups, detected in 89.1% of the dMMR cases and 83.1% of the pMMR cases. However, defects in the PI3K (68.6% vs. 19.6%, $P < 0.001$), RTK-RAS (69.2% vs. 54.2%, $P = 0.004$), and TGF β (32.7% vs. 14.2%, $P = 0.001$) pathways were significantly enriched in the dMMR CRCs compared with their frequencies in the pMMR CRCs, and aberrations in the TP53 pathway (55.8% vs. 85.3%, $P < 0.001$) were significantly more frequent in the latter cases (Fig. 2b).

Mutual relationships among key genes in the dMMR and pMMR CRCs

Compared to those in the pMMR CRCs, genes in the WNT (83.3% vs. 40.4%, $P < 0.001$) and PI3K (23.7% vs. 4.4%,

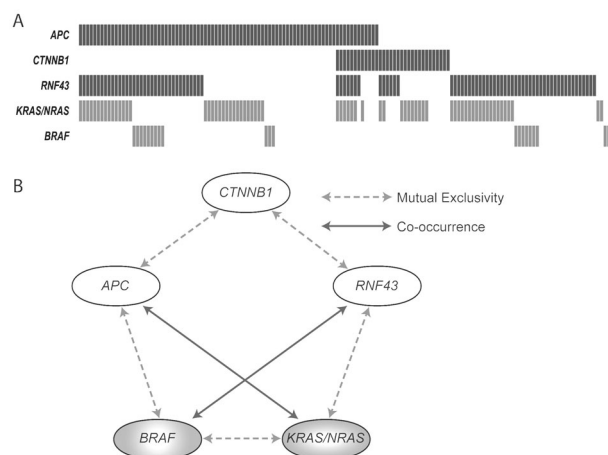


Fig. 3 Mutation profile and mutual relationship of genes in WNT and RTK-RAS pathways in dMMR CRCs with MLH1 hypermethylation. (a) Mutation profile of key WNT and RTK-RAS pathway genes in the dMMR CRCs with MLH1 hypermethylation. Columns present individual cases of dMMR CRCs with MLH1 hypermethylation. APC, CTNNB1, RNF43, KRAS/NRAS and BRAF mutations are listed in rows. (b) Mutual relationships of key WNT and RTK-RAS pathway genes in the dMMR CRCs with MLH1 hypermethylation.

$P < 0.001$) pathways in the dMMR CRCs were more likely to harbor two or more mutations. Specifically, of the mutations in the key genes in the WNT pathway, *CTNNB1* mutations were always mutually exclusive of *APC* and *RNF43* mutations (Fig. 3a), while *AXIN2* mutations were often incompatible with *APC* mutations but coexisted with *RNF43* mutations. *PIK3CA* and *PIK3R1*, two key components of the PI3K pathway, displayed neither a significant coexistent nor incompatible relationship. The majority of mutations of genes within the RTK-RAS and TGF β pathways exhibited mutually exclusive patterns. Notably, all eight dMMR tumors bearing two or more RTK-RAS pathway alterations had a *BRAF* V600E or *KRAS/NRAS*-activating mutation and an additional oncogenic *ERBB3* mutation (G284R or V104M).

We further analyzed the coordination among key genes involved in WNT and RTK-RAS signaling, the two pathways with the most frequently mutated genes in CRCs. Among the dMMR CRCs, 19 of 22 (86.4%) *BRAF* mutations were carried concurrently with an *RNF43* mutation, while 46 of 68 (67.6%) *KRAS/NRAS* mutations were carried concurrently with an *APC* mutation (Fig. 3a). A statistically significant coexistence relationship between *BRAF* and *RNF43* mutations ($P = 0.016$) and between *KRAS/NRAS* and *APC* mutations ($P = 0.006$) was observed. However, neither the *BRAF* and *APC* mutations ($P = 0.002$) nor the *KRAS/NRAS* and *RNF43* mutations ($P = 0.001$) exhibited mutually exclusive patterns. The mutual relationship between *CTNNB1*, *APC*, *RNF43*, *KRAS/NRAS*, and *BRAF* mutations in the dMMR group is illustrated in Fig. 3b. The

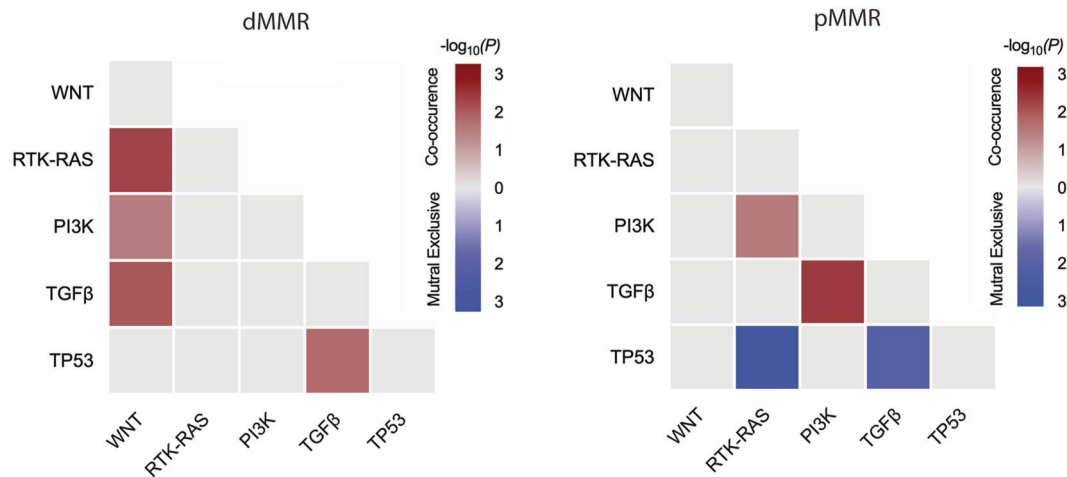


Fig. 4 Mutual relationships among the WNT, RTK-RAS, PI3K, TGF- β , and TP53 pathways in the dMMR and pMMR CRC cases. Co-occurrence and mutual exclusivity were identified using the Chi-

square test or Fisher's exact test. The significance level is encoded in color representing $-\log_{10}(P)$ value.

RNF43 mutation was very uncommon in the pMMR group (13/225, 5.8%) and detected in two of nine (22.2%) *BRAF* mutant pMMR cases. There was a tendency for *RNF43* and *BRAF* mutations to coexist in the pMMR CRCs, but a level of significance was not reached ($P = 0.088$, Fisher's exact test).

Mutual relationships between signaling pathways in the dMMR and pMMR CRCs

The mutual relationships of the WNT, RTK-RAS, PI3K, TGF- β , and TP53 pathways are shown in Fig. 4, displaying remarkable differences between the dMMR and pMMR CRCs. Among the dMMR tumors, all the WNT pathway gene aberrations co-occurred with defects in other cancer-driving signaling pathways, including RTK-RAS ($P = 0.008$), TGF- β ($P = 0.013$), and PI3K ($P = 0.043$). In addition, the TGF- β pathway was generally altered concurrently with TP53 pathway alterations ($P = 0.024$). However, pMMR tumors showed co-alterations in the PI3K and RTK-RAS pathways ($P = 0.038$) and the PI3K and TGF- β pathways ($P = 0.006$). No mutually exclusive pattern was observed for the dMMR group genes, whereas TP53 pathway deficiency genes were not compatible with RTK-RAS ($P = 0.002$) or TGF- β ($P = 0.012$) pathway alterations in the pMMR group.

Mutation profile of the dMMR CRCs with *MLH1* hypermethylation

The 156 dMMR CRC tumors were categorized into four groups: the Lynch syndrome-associated group consisted of 57 tumors harboring pathogenic/likely pathogenic germline mutations in one of the four MMR genes or combined *EPCAM-MSH2* deletions (57/156, 36%); the *MLH1*-

hypermethylated group (*MLH1*^{me+} group) consisted of 67 dMMR cases with lost *MLH1*/*PMS2* expression and *MLH1* promoter hypermethylation but no *MLH1* germline mutation (67/156, 43%), and this group was further divided into the “*MLH1*^{me+} with fusion” ($n = 15$) subgroup and the “*MLH1*^{me+} without fusion” ($n = 52$) subgroup according to the presence or absence of oncogenic RTK gene rearrangements; and the Lynch-like group consisted of the remaining patients with neither MMR gene germline mutations nor *MLH1* promoter hypermethylation (32/156, 21%) (Supplementary Fig. 1). As detailed in Fig. 5 and summarized in Fig. 6, apart from the enrichment of the *BRAF* V600E mutation (38.8%), the *MLH1*^{me+} group was found to harbor a significantly higher frequency of *RNF43* mutations (73.1% vs. 43.8%, $P < 0.001$) but much lower frequencies of *KRAS* (28.1% vs. 61.7%, $P < 0.001$), *APC* (43.3% vs. 63.6%, $P = 0.011$), *CTNNB1* (9.0% vs. 29.2%, $P = 0.001$), *FAM123B* (13.4% vs. 28.1%, $P = 0.025$), and *SOX9* (17.9% vs. 40.0%, $P = 0.012$) mutations compared to those in the *MLH1*-unmethylated dMMR CRCs (*MLH1*^{me-} group). Regarding co-alteration patterns, 19 cases of *BRAF*/*RNF43* comutated tumors were exclusively detected in the *MLH1*^{me+} group, whereas 46 cases of *RAS*/*APC* comutated tumors were found mainly in the *MLH1*^{me-} group (35/46, 76.1%), especially among tumors associated with Lynch syndrome (27/46, 58.7%). After controlling for *BRAF* mutation status, the *MLH1*^{me+} phenotype was independently associated with an increased incidence of *RNF43* mutations (95% CI 1.76–6.91; $P < 0.001$) and a decreased incidence of *CTNNB1* mutations (95% CI 0.09–0.62; $P = 0.003$). At the signaling pathway level, no significant differences in the WNT, RTK-RAS, PI3K, TGF- β , TP53, or DDR pathways were observed between the *MLH1*^{me+} and *MLH1*^{me-} groups.

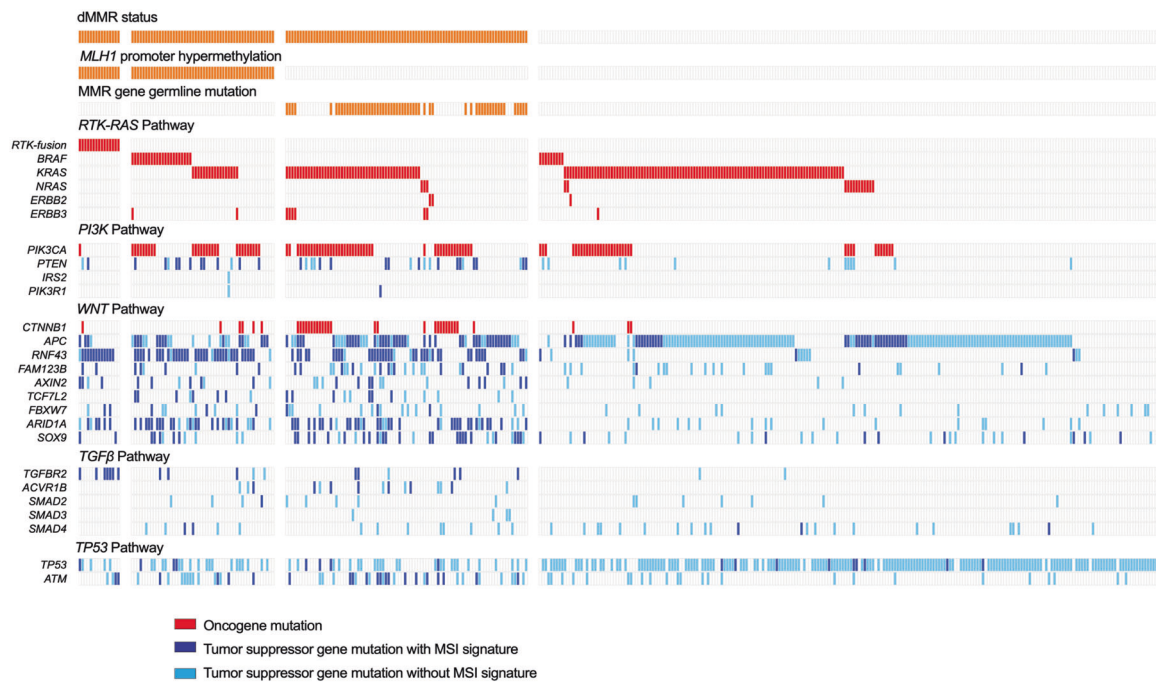


Fig. 5 Mutation profile of key genes in the dMMR and pMMR CRCs. Columns represent individual cases sorted by MMR status and dMMR subgroups. Tracks indicate dMMR status, *MLH1* promoter hypermethylation status, MMR gene germline mutation status and RTK-RAS, PI3K, WNT, TGFβ, and TP53 pathway gene mutations.

Individual genes are listed in rows. Mutations affecting tumor suppressor genes are classified according to whether they bear the MSI signature (insertions or deletions affecting homopolymer sequences). dMMR deficient mismatch repair, pMMR proficient mismatch repair, MSI microsatellite stability.

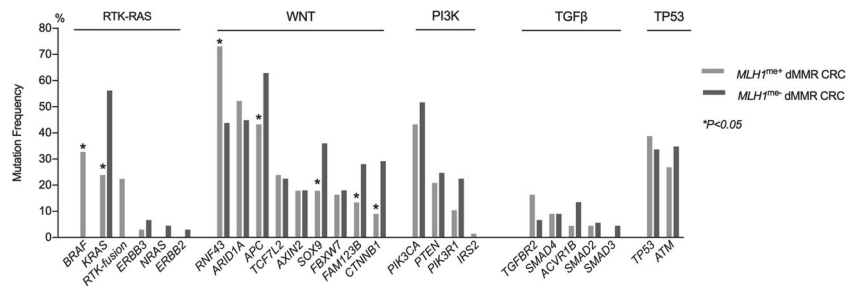


Fig. 6 Comparison of gene mutation frequencies in the *MLH1*^{me+} dMMR CRCs and *MLH1*^{me-} dMMR CRCs. *MLH1*^{me+} hypermethylated *MLH1* promoter, *MLH1*^{me-} *MLH1* promoter not

hypermethylated. Asterisk (*) significant difference in mutational prevalence (Chi-square test or Fisher’s exact test, $P < 0.05$).

Mutation profile of the dMMR CRCs harboring *MLH1* hypermethylation with RTK fusion genes

Fifteen of sixty-seven (22.4%) *MLH1*^{me+} dMMR CRCs were identified with oncogenic RTK gene rearrangements, including *NTRK1* ($n = 7$), *NTRK3* ($n = 3$), *ALK* ($n = 3$), and *RET* ($n = 2$), and were classified as the “*MLH1*^{me+} with fusion” subgroup. As shown in Fig. 5, none of these “*MLH1*^{me+} with fusion” tumors harbored alterations in other key driver genes in the RTK-RAS pathway, including *BRAF*, *KRAS*, *NRAS*, *ERBB2*, or *ERBB3*. Mutations affecting WNT pathway components were found in 12 cases (80.0%), mainly including inactivating mutations in tumor suppressor genes, *RNF43* ($n = 12$), *ARID1A* ($n = 8$),

APC ($n = 4$), *AXIN2* ($n = 4$), *FBXW7* ($n = 3$), *FAM123B* ($n = 2$), *SOX9* ($n = 2$), and *TCF7L2* ($n = 1$), except for one activating mutation in *CTNNB1*. Mutations in the PI3K signaling pathway (*PIK3CA*, *PIK3R1*, and *PTEN*) were identified in three tumors. The incidence of PI3K pathway aberrations was significantly lower in the “*MLH1*^{me+} with fusion” subgroup tumors than it was in the other dMMR tumors (20% vs. 73.8%, $P < 0.001$) or *MLH1*^{me+} tumors without RTK fusions (20% vs. 73.1%, $P = 0.001$). In addition, all TGFβ pathway aberrations in the “*MLH1*^{me+} with fusion” subgroup were found to be *TGFBR2* frameshift mutations bearing MSI signature, which were significantly more prevalent than those in other dMMR tumors (46.7% vs. 7.8%, $P = 0.002$).

Mutation profile of the Lynch-like group of dMMR CRCs

Among the 32 tumor cases in the Lynch-like subgroup, 5 cases had 2 different heterozygous deleterious mutations in the same MMR gene (3 in *MLH1*, 1 in *MSH2*, and 1 in *MSH6*), and 5 cases had a single deleterious mutation in genes accompanied with associated LOH (3 in *MLH1*, 1 in *MSH2*, and 1 in *PMS2*), findings consistent with “double somatic mutations” (10/32, 31.3%). Fourteen cases were found to have only one deleterious mutation with a possible LOH explaining their IHC staining and were considered to harbor “possible double somatic mutations” (14/32, 43.8%). The other eight cases (five with *PMS2* loss and three with *MSH2/MSH6* loss) remained unresolved. Tumors in the Lynch-like group displayed no *BRAF* mutations and exhibited a remarkably lower frequency of *RNF43* (40.6% vs. 73.1%, $P = 0.002$) mutations but a much higher frequency of *CTNNB1* mutations (37.5% vs. 9.0%, $P = 0.001$) compared with the *MLH1*^{me+} dMMR group. No significant differences in the mutation profiles of key genes were detected in the Lynch-like group and the Lynch group of dMMR CRCs.

Discussion

In this study, we conducted a detailed molecular genetic analysis of a retrospective cohort comprised of 156 dMMR CRC and 225 pMMR CRC cases. Through the use of CGP, the gene mutation prevalence and co-alteration relationship of genes in key CRC oncogenic pathways were profiled, including the WNT, RTK-RAS, PI3K, TGF- β , TP53, and DDR pathways. Based on MMR germline mutations, *MLH1* hypermethylation status and the presence of RTK fusion, the “*MLH1*^{me+}” and “*MLH1*^{me+} with fusion” subgroups of the dMMR CRCs were identified and their molecular features further analyzed. We documented the characteristic mutation spectrum, as well as the pattern of gene co-occurrence and mutual exclusivity in the dMMR CRCs, with an emphasis on the molecular heterogeneity among dMMR subgroups.

Our data showed noticeable differences in the composition of most recurrently altered genes and their mutation frequencies in the dMMR and pMMR CRCs. In agreement with previous reports [17, 29], mutations in *RNF43* and *TCF7L2*, both of which acting as key negative regulators in the WNT signaling pathway, were highly prevalent in our dMMR cohort but uncommon in the pMMR cohort. Of the well-known CRC-driver genes, *KRAS* and *NRAS* were frequently mutated regardless of MMR status, indicating their universally important role in CRC carcinogenesis. *PIK3CA* mutations, which were reported to be moderately enriched in dMMR CRCs in early studies [30, 31], were also found at

a higher frequency in our dMMR group. However, *TP53* and *APC* mutations, although frequently detected in both groups, were far more common in the pMMR group, a finding similar to that for the TCGA cohort [17]. In particular, the remarkable preponderance of *TP53* mutations in pMMR tumors, along with the lack of an MSI signature, suggested that *TP53* is unlikely to be a main mutational target for deficient MMR.

Notably, our study revealed a considerably high mutational incidence in multiple components of the DDR system, including *ARID1A*, *ATM*, and *BRCA2*, which has not been highlighted in previous studies of dMMR CRCs. As previously demonstrated [32, 33], *ARID1A*, *ATM*, and *BRCA2* are all essential tumor suppressor genes that directly participate in the HRR of DNA double-strand breaks. Accordingly, we also showed that the HRR pathway was the most frequently defected one in DDR system apart from MMR pathway. These findings indicated that coordinated alterations of the HRR and MMR pathways might play an important role in CRC carcinogenesis, highlighting the synergistic effect of the DDR system. Both germline and somatic deleterious *BRCA* mutations have been established as indicators for the application of poly (ADP-ribose) polymerase (PARP) inhibitors in ovarian cancer. The increased frequency of *BRCA2* mutations documented in our dMMR cohort, which largely contributed to MSI-related aberrations, implied the potential application of PARPi therapy in a portion of dMMR CRC cases and hinted that MMR deficiency might precede and lead to *BRCA2* alterations. Moreover, there is growing appreciation that, in multiple solid malignancies, such as prostate cancer, bladder cancer, and triple-negative breast cancer, the dysfunction of other essential HRR proteins might reflect a response to PARPis [34, 35]. In a preclinical study, CRC cell lines carrying inactivating *ATM* mutations were found to exhibit increased sensitivity to olaparib [36]. Therefore, our finding that HRR-related genes were recurrently affected targets in dMMR CRC cases justifies further exploration of HRR inhibition therapy as a promising anticancer strategy in these tumors.

We showed that the WNT pathway was commonly altered in CRCs regardless of MMR status, supporting the supposition that it has an indispensable role in CRC oncogenesis. In particular, dMMR CRCs were more likely to harbor multiple WNT pathway alterations with complicated mutual relationships. Considering that *CTNNB1* (β -catenin) acts as the main effector of the canonical WNT pathway and is negatively regulated by *APC* and *RNF43* at different levels, the mutual exclusivity of the *CTNNB1* mutation and the *APC* or *RNF43* mutation found in the dMMR cohort was predictable. The RTK-RAS pathway is also frequently hyperactivated in CRCs, endowing it with key prognostic and predictive relevance. Despite the general mutually exclusive pattern within this pathway, we noticed that

ERBB3 mutations often co-occurred with other RTK-RAS oncogenic mutations in the dMMR CRCs. This observation was also evidenced by a preclinical study suggesting the inadequacy of isolated *ERBB3* mutations for oncogenic transformation [37]. Therapeutically targeting the PI3K pathway has become an area of intensive studies of multiple solid tumors [38], and an oral PI3K inhibitor has recently been approved by the US Food and Drug Administration for advanced breast cancers with *PIK3CA* mutations. Our finding of remarkably increased PI3K pathway aberrations in the dMMR CRCs thus leads to the consideration of including PI3K-targeted therapy in anticancer regimens for these tumors. The preferential coexistence of the PI3K and WNT pathways in our dMMR cohort further indicated the promising application of a PI3K inhibition strategy, as shown in a functional study whereby *PI3KCA* and *APC* comutated CRCs were more likely to respond to dual PI3K/mTOR inhibitors [39].

We further explored the intricate interaction between key genes involved in the WNT and RTK-RAS pathways. In addition to the preferential co-occurrence of *RNF43* and *BRAF* mutations reported in a previous study [40, 41], we presented a novel finding showing a high prevalence of concurrent *APC* and *RAS* mutations in dMMR CRCs. We additionally showed the mutually exclusive relationship between *RNF43* and *RAS* mutations and between *APC* and *BRAF* mutations. More specifically, *RNF43/BRAF* comutations and *APC/RAS* comutations were found to be enriched in sporadic and hereditary dMMR CRC cases, respectively. Both clinical and functional findings have suggested that concurrent *RNF43* and *BRAF* mutations generally indicate the serrated neoplasia pathway leading to the majority of sporadic dMMR CRCs [41, 42]. On the other hand, *APC* and *RAS* mutations collaborate in the conventional adenoma pathway, which accounts for carcinogenesis in the majority of Lynch syndrome-associated CRCs [43]. Taken together, our data provide molecular evidence that dMMR CRC oncogenesis follows separate pathways with divergent mechanisms of RTK-RAS and WNT activation. The predisposition for a specific set of driver mutations caused by intrinsic mutational processes has been suggested in various types of cancers [44]. Therefore, it is rational to assume that the comprehensive interplay between oncogenic events across pathways, which was found to be more remarkable among dMMR CRCs in our cohort, might reflect a distinctive driver mutation profile conferred by MMR defects.

The differential involvement of key WNT pathway genes in the *MLH1*^{me+} and *MLH1*^{me-} dMMR CRCs was investigated by previous studies [41]. However, among *MLH1*^{me+} tumors, whether the increased *RNF43* mutation frequency was directly linked to the *MLH1*^{me+} phenotype or *BRAF* mutational status was not previously elucidated. Through

logistic regression analysis, we demonstrated that *MLH1*^{me+} was an independent factor associated with prevalent *RNF43* mutations in dMMR CRCs, but not *BRAF* mutations. This association was further supported by the fact that *RNF43* mutations were also relatively frequent in the *MLH1*^{me+} tumors with wild-type *BRAF*, including those harboring RTK fusion genes. It should be noted that *RNF43* mutations were also detected in a considerable proportion of the *MLH1*^{me-} dMMR tumors, although they were significantly less frequent than in the *MLH1*^{me+} tumors, hinting at a possible correlation between *RNF43* defects and MMR deficiency not caused by *MLH1* hypermethylation. Moreover, the majority of *RNF43* mutations in both the *MLH1*^{me+} and *MLH1*^{me-} dMMR tumors were found to be associated with MSI. All these results suggested that MMR deficiency, especially that caused by *MLH1* hypermethylation, might be the direct cause of *RNF43* mutations. In addition, *RNF43* mutations in our pMMR group, which were uncommon and did not affect the MSI signature, exhibited a tendency to coexistence with *BRAF* mutations. This finding suggests a weak association between *RNF43* and *BRAF* mutations in pMMR CRCs, albeit to a much lesser extent than their association in the dMMR CRCs. Referring to a previous study [41], these rare *RNF43/BRAF* comutated pMMR cases probably indicate germline *RNF43* mutation carriers with a serrated polyposis phenotype. To summarize, our findings revealed that the mutation of *RNF43*, which might represent a dysfunctional negative feedback mechanism of the WNT pathway, was an indispensable yet relatively late event in *MLH1*^{me+} dMMR CRC oncogenesis and was independently correlated with *MLH1* hypermethylation but not *BRAF* mutation. It should be noted that *RNF43* p. G659Vfs*41, which was the most common *RNF43* mutant and accounted for 60% of the *RNF43* mutations in our dMMR cohort, was suggested to be fully functional in inhibiting WNT signaling in an in vitro study [45]. The significant role of mutated *RNF43* in the pathogenesis of *MLH1*^{me+} tumors might not be entirely substantiated by the present data and awaits further exploration. In addition, we also observed decreased incidence of *KRAS* mutation and that of other WNT signaling components (*CTNNB1*, *APC*, *FAM123B*, and *SOX9*) in *MLH1*^{me+} tumors, reflecting the characteristic mutational spectrum of this subgroup.

A recent functional study suggested that RTK fusion genes might be alternative drivers of the sessile serrated pathway in CRC carcinogenesis [46]. Within our cohort, the “*MLH1*^{me+} with fusion” subgroup shared some genetic features typically presented by *MLH1*^{me+} dMMR CRCs but also displayed intricate differences (Supplementary Fig. 2). Given the established fact that oncogenic mutations within the RTK-RAS pathway are generally mutually exclusive, it was not surprising to find that other RTK-RAS oncogenic alterations were excluded from the “*MLH1*^{me+} with fusion”

subgroup. Intriguingly, although PI3K pathway aberration was found to be highly prevalent in the dMMR CRCs, its incidence was significantly decreased in the “*MLHI*^{me+} with fusion” subgroup. This paradox might be partially explained by the fact that the constitutive activation of tyrosine kinase by gene rearrangements was able to simultaneously induce downstream RAS and PI3K pathways through the recruitment of various adapter proteins [47]. In those particular cases carrying concurrent RTK fusion and PI3K pathway aberration, we speculated the altered PI3K pathway might represent a nonoverlapping mechanism besides RTK hyperactivation, giving rise to downstream malignant transformation events. We also noticed that TGFβ pathway inactivation in the “*MLHI*^{me+} with fusion” subgroup was solely caused by *TGFBR2* truncation mutations.

“Lynch-like” dMMR CRCs have long been recognized as an ill-defined subgroup. Our results revealed that the oncogenic pathway alteration profile of “Lynch-like” tumors was more similar to that of Lynch syndrome-associated tumors than to that of *MLHI*^{me+} tumors. In addition, we confirmed that the majority of cases in the “Lynch-like” subgroup can be explained by inactivation of somatic MMR genes that might be indicators of low cancer risk that do not necessarily need intensive screening or surveillance.

In the present study, the *KRAS* mutation frequency of dMMR CRCs was higher than it was in previous reports on Western populations [17, 48] but similar to that in studies based on the Chinese population [49, 50], which might be partially explained by the genetic differences among ethnic groups. However, our results need further validation in a larger multicenter cohort.

In summary, our comprehensive molecular study revealed a significant difference in the gene mutational spectrum and signaling pathway interaction pattern between dMMR and pMMR CRCs. Through detailed profiling of the molecular features among dMMR subgroups, we presented a differential mechanism and complex mutual relationship of the oncogenic events in the MSI and serrated pathways of CRC tumorigenesis (Supplementary Fig. 2) [40, 41]. This molecular heterogeneity probably underlies the different response to adjuvant therapy of dMMR CRCs and justifies individualized therapy targeting dMMR subgroups.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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