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Somatic hypermethylation of *MSH2* is a frequent event in Lynch Syndrome colorectal cancers

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Abstract

Heritable germline epimutations in *MSH2* have been reported in a small number of Lynch Syndrome families which lacked germline mutations in the *MSH2* gene. It is not known whether somatic *MSH2* methylation occurs in *MSH2* mutation-positive Lynch Syndrome subjects or sporadic colorectal cancers (CRCs). Therefore, we determined the methylation status of the *MSH2* gene in 268 CRC tissues, including 222 sporadic CRCs and 46 tumors that did not express *MSH2*. We also looked for microsatellite instability (MSI), germline mutations in the *MSH2* and *EpCAM* genes, somatic mutations in *BRAF* and *KRAS*, and the CpG island methylator phenotype (CIMP). We observed that somatic *MSH2* hypermethylation was present in 24% (11 of 46) of *MSH2*-deficient (presumed Lynch Syndrome) tumors, while no evidence for *MSH2* methylation existed in sporadic CRCs (MSI and MSS) or normal colonic tissues. Seven of 11 (63%) patients with *MSH2* methylation harbored simultaneous pathogenic germline mutations in the *MSH2* gene. Germline *EpCAM* deletions were present in 3 of 4 patients with *MSH2* methylation but without pathogenic *MSH2* germline mutations. The mean methylation scores at CIMP-related markers were significantly higher in Lynch Syndrome tumors with *MSH2* methylation than *MSH2*-unmethylated CRCs. In conclusion, our data provide evidence for frequent *MSH2* hypermethylation in Lynch Syndrome tumors with *MSH2* deficiency. *MSH2* methylation in this subset of individuals is somatic, and may serve as the ‘second hit’ at the wild type allele. High levels of aberrant methylation at CIMP-related markers in *MSH2* methylated tumors raises the possibility that *MSH2* is a target susceptible to aberrant methylation in Lynch Syndrome.

Keywords

Colon cancer; *MSH2*; Lynch Syndrome; DNA methylation; *EpCAM*; DNA mismatch repair

Introduction

Lynch Syndrome (previously called hereditary non-polyposis colorectal cancer or HNPCC) is an autosomal dominant colorectal cancer susceptibility syndrome characterized by

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germline mutations in DNA mismatch repair (MMR) genes, most frequently in *MLH1* and *MSH2*, and less often in *MSH6* and *PMS2* (1–3). Mutational inactivation of MMR genes lead to insufficient DNA repair and the development of tumors with high levels of microsatellite instability (MSI-H), which is a characteristic feature of >95% of Lynch Syndrome associated colorectal cancers (CRC) (4,5). Patients with Lynch Syndrome typically demonstrate a germline mutation and somatic inactivation of the wild type allele of the relevant MMR gene through a second event that is either a mutation or deletion of the wild-type allele.

The *MLH1* gene is methylated in ~12% of sporadic CRCs (6), giving rise to a MSI-H phenotype with similar clinico-pathological features as hereditary tumors (7–11). It is believed that these sporadic MSI CRCs evolve through the CpG island methylator phenotype (CIMP) pathway, in which *MLH1* is one of multiple different targets of transcriptional inactivation (12–14).

Recent discoveries have suggested a novel paradigm, in which the DNA MMR genes *MLH1* and *MSH2* can be targets of ‘germline methylation’ in some individuals with Lynch Syndrome (15–18). The first evidence for this came from studies in which *MLH1* was found to be methylated in the peripheral blood and other germline tissues in Lynch Syndrome patients who did not carry germline *MLH1* mutations (16,17,19). More recently, heritable germline epimutations in *MSH2* were reported in a few mutation-negative Lynch Syndrome families (18,20). Subsequent studies have revealed that germline deletions at the 3’-end of the *EpCAM* gene (formerly called *TACSTD1*), located immediately upstream of *MSH2*, is the cause of this heritable somatic epimutation (21).

In spite of the growing interest in ‘germline’ epigenetic regulation of MMR genes in Lynch Syndrome CRC, to the best of our knowledge, no study has investigated the role of ‘somatic’ *MSH2* promoter methylation in the pathogenesis of sporadic MSI and *MSH2*-deficient Lynch Syndrome tumors. In view of this gap in understanding, we studied a group of 46 *MSH2*-deficient Lynch Syndrome CRCs for germline mutations and the methylation status of the *MSH2* gene, and deletions in the *EpCAM* gene. In addition, we studied *MSH2* methylation in a cohort of 222 sporadic CRCs, which included 15 sporadic MSI tumors. Herein, we report that somatic *MSH2* methylation is frequent in Lynch Syndrome CRCs, and may constitute the ‘second hit’ required to inactivate the wild-type *MSH2* allele. Furthermore, we found excessive methylation at CIMP-related loci in *MSH2* methylated Lynch Syndrome CRCs, which suggests that *MSH2* is a frequent susceptibility target of aberrant methylation in the colon.

Materials and Methods

Tissue specimens

This study analyzed a cohort of 268 CRCs, which included 222 sporadic cancers and 46 Lynch Syndrome tumors. All 222 sporadic CRCs, which included 15 sporadic MSI cancers were enrolled at the Okayama University Hospital, Okayama, Japan. Tumor tissues from 46 Lynch syndrome CRCs lacking *MSH2* expression were obtained from Heidelberg University, Heidelberg, Germany (22). The patients were classified to have a Lynch syndrome associated CRC if either a pathogenic germline mutation was identified in the *MSH2* gene or if the patients fulfilled Bethesda/Amsterdam criteria and presented with one or more MSI-H CRCs that lacked expression of the *MLH1* or *MSH2* protein by immunohistochemistry. Similarly, patients deemed to have a sporadic MSI-positive CRC when they failed to fulfil criteria for hereditary cancer, but showed loss of *MLH1* protein expression and associated methylation of the promoter region (Supplementary Table 1). In the cohort of 46 *MSH2*-deficient CRCs, DNA was available from 35 cases for germline

mutation analysis of the *MSH2* gene. Germline deletion analysis at the 3'- end of the *EpCAM* gene was performed on all samples that showed *MSH2* methylation (n=11). Patients provided informed consent for use of their tissues, and Institutional Review Boards of both institutions approved this study.

Microsatellite instability analysis

MSI analysis was performed by examination of the NCI-workshop panel of five markers, which included two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeat (D2S123, D5S346 and D17S250)(23). Tumors showing allelic shifts in $\geq 2/5$ markers were classified as MSI-H (hereupon referred to as "MSI"), and the rest were classified as MSS. Utilizing this criterion, all 46 *MSH2*-deficient CRCs were MSI. Of the 222 sporadic CRCs, 15 cases were MSI and the remaining 207 cancers were MSS.

Immunohistochemical staining (IHC) of MLH1 and MSH2 proteins

IHC staining was performed to determine protein expression for the MLH1 and MSH2 proteins in all Lynch syndrome and sporadic MSI cases. IHC staining was performed on formalin-fixed paraffin-embedded tissues, using the tyramide signal amplification biotin system (PerkinElmer, Boston, MA). Briefly, after deparaffinization and rehydration, antigen retrieval was achieved by immersing the tissue sections in citrate buffer (pH 6.0) and exposed to microwave irradiation for 20 minutes. Thereafter, tissue sections were blocked for endogenous peroxidase in phosphate-buffered saline containing 3% H₂O₂, and the sections were incubated for 3 h with a monoclonal antibody for hMLH1 (clone G 128-728, 1/100, BD PharMingen, San Diego, CA) or hMSH2 (clone G219-1129, 1/3,000, BD PharMingen). Negative control slides were incubated with phosphate buffer instead of a specific antibody. This step was followed by further incubations in secondary antibody (Vector Laboratories, Burlingame, CA), streptavidin-peroxidase and biotinyl tyramide. The final brown coloration for both MMR proteins was developed using diaminobenzidine as a chromogen, and hematoxylin as a nuclear counterstain. Sections with obvious nuclear staining were deemed positive. Tumor tissues were considered negative only when there was a clear evidence for positive staining in the surrounding non-neoplastic tissues including normal colonic epithelium, lymphocytes or stromal cells.

Germline mutation analysis of the *MSH2* gene

MSH2 germline mutation analyses were performed by initial prescreening for mutations by denaturing high performance liquid chromatography, followed by mutation confirmation through direct sequencing as described previously (22,24). A systematic search for large genomic deletions was performed using multiplex ligation-dependent probe amplification (MLPA) according to the manufacturer's protocol (MRC-Holland, Amsterdam, The Netherlands) (22).

Sodium bisulfite modification and CIMP analyses

Genomic DNA from tumor tissues and the corresponding normal mucosa of all 46 *MSH2*-deficient Lynch Syndrome CRCs, 15 sporadic MSI CRCs, and 207 sporadic MSS CRCs was available for methylation analyses. Genomic DNA was bisulfite modified to convert all the unmethylated cytosine residues to uracils. Briefly, 0.5–2.0 μ g of DNA was denatured with NaOH, treated with sodium bisulfite, and purified using the Wizard DNA Clean-up System (Promega, Madison, WI). The methylation status of *MLH1*, *p16^{INK4a}*, *p14^{ARF}*, *MINT1*, *MINT2*, and *MINT31* CIMP markers was evaluated by Combined Bisulfite Restriction Analysis (COBRA) as described previously (14). Following densitometric quantification of methylated and unmethylated bands, tumors with $\geq 5\%$ methylation at each marker were considered methylation-positive, while tumors with low background levels of methylation,

which may be present in some normal appearing colorectal mucosa (<5%), were defined as methylation-negative as described previously (14).

Methylation analysis of the MSH2 promoter region

Methylation status of the *MSH2* promoter CpG island was investigated by COBRA and bisulfite sequencing procedures. Supplementary Table 2 lists the primer sequences and PCR conditions for both methodologies. PCR reactions for COBRA were carried out on bisulfite-modified template DNA in a 25 μ l PCR mixture containing 12.5 μ l of HotStarTaq Master Mix kit (Qiagen, Valencia, CA), 0.5 μ M of each PCR primer, and approximately 25 ng of bisulfite-modified DNA. The PCR products were digested with *TaqI* or *HpyCH4IV* (New England Biolabs Inc, Ipswich, MA) at 65°C or 37°C for 16h, respectively. The digested DNA was separated on 3% agarose gels in 1 \times TAE buffer and stained with ethidium bromide. A Gel Logic 200 Imaging System (Eastman Kodak Co., Rochester, NY) was used to perform densitometric analyses on all gels. Band intensities were quantified using Kodak 1D analysis software (Eastman Kodak Co., Rochester, NY). The methylation levels (ratios of methylated to unmethylated DNA) were determined from the relative intensities of cut and uncut PCR products to quantify methylation. As with the CIMP markers described previously, tumors with \geq 5% *MSH2* methylation were considered methylation-positive, while tumors with low background levels of *MSH2*-methylation (<5%) were defined as methylation-negative. Human normal colonic DNA treated with *SssI* methylase (New England Biolabs Inc) was used as a positive control for methylated alleles, whereas DNA from normal lymphocytes was used as a control for unmethylated alleles. Water was used as a negative PCR control to monitor for PCR contamination.

To confirm the methylation profiles obtained by COBRA, bisulfite sequencing for the *MSH2* promoter region was performed in a subgroup of *MSH2* methylated and unmethylated tumors. For bisulfite sequencing, nested PCR was performed in a 25 μ l PCR mixture containing 12.5 μ l of HotStarTaq Master Mix kit (Qiagen) and appropriate concentrations of PCR primers (primer sequences are shown in Supplementary Table 2). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced on an ABI 3100-Avant DNA sequencer.

EpCAM deletion analysis

The *EpCAM* gene was screened for the deletion of exons 3, 8 and 9 using the SALSA MLPA Kit P072-B1 from MRC-Holland (Amsterdam, The Netherlands) according to the manufacturer's instructions. DNA from a healthy donor without a deletion at the 3' end of *EpCAM* was used as a negative control for MLPA analysis.

BRAF and *KRAS* mutation analyses

Direct sequencing was performed to identify *BRAF* exon15 (V600E) mutations and *KRAS* exon 2 (codon 12/13) mutations. PCR for the *BRAF* and the *KRAS* genes was carried out in a 25 μ l PCR mixture containing 12.5 μ l of HotStarTaq Master Mix kit (Qiagen) with concentrations of primers, listed in Supplementary Table 1, as previously described (14). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced on an ABI 3100-Avant DNA sequencer.

Statistical analyses

The methylation status of *MSH2* and other loci as determined by COBRA was analyzed as a categorical variable (methylated = methylation level \geq 5%, unmethylated = methylation level <5%). *MSH2*-deficient CRCs were divided into subgroups according to *MSH2* methylation status, and were analyzed for potential associations with a number of clinico-pathological

and epigenetic parameters using the X2 test. Methylation scores were calculated based upon the total number of loci methylated at CIMP markers. The differences in mean methylation scores between *MSH2* methylated and unmethylated cases were analyzed by the Kruskal-Wallis test. If the Kruskal-Wallis test indicated differences among various CRC subgroups, further pair-wise comparisons for each of the subgroups were performed using the Steel-Dwass test, which is a non-parametric multiple comparison method. All reported *P* values are 2-sided and a *P* <.05 was considered statistically significant.

Results

***MSH2* is frequently methylated in Lynch Syndrome, but not sporadic, CRCs**

In this study, using COBRA, we investigated the *MSH2* methylation status in tumor and non-neoplastic tissues from 46 *MSH2*-deficient cancers, 15 sporadic MSI patients, and 207 MSS CRCs. The COBRA for *MSH2* was designed and optimized to examine methylation levels of a CpG site located at -73 bp from the transcription start site (TSS) of the *MSH2* promoter (Figure 1A). This CpG site was identified through bisulfite sequencing analysis of a larger region of the *MSH2* promoter, and is within the same segment of the promoter reported to be methylated in a recent publication (18). The methylation levels were quantitated, and the lower limit of measurable methylation was $\geq 1\%$ (Figures 1B and 1C).

Among the group of 46 *MSH2*-deficient Lynch syndrome CRCs, 11 (24%) cases showed somatic hypermethylation in the *MSH2* promoter (Figure 1D and Table 1). On the other hand, none of the 222 sporadic tumors that included both sporadic MSI cancers and MSS tumors demonstrated *MSH2* promoter methylation. Similarly, we did not see any evidence for *MSH2* methylation in the entire collection of matching normal mucosal DNA from the 46 *MSH2*-deficient CRC patients.

To further support and confirm the methylation data obtained by COBRA, we next performed direct bisulfite sequencing in a subset of 13 *MSH2*-deficient CRCs (9 cases with *MSH2* methylation and 4 cases without *MSH2* methylation: Figures 1D). This approach allowed us to further confirm the methylation profile of the 14 CpG sites that are located between the -93bp and +32 bp region of the *MSH2* promoter. All 9 CRCs which showed methylation by COBRA also demonstrated widespread *MSH2* promoter methylation when analyzed by bisulfite sequencing (Figure 1D). On the contrary, congruent with our COBRA results, none of the *MSH2*-proficient tumors showed any evidence for *MSH2* methylation at any of the CpG dinucleotides within the promoter's CpG island.

***MSH2* promoter methylation may constitute the second hit in *MSH2*-deficient Lynch Syndrome CRCs**

We next questioned the relevance of *MSH2* methylation in the context of other genetic alterations in MMR-deficient CRCs. Since evidence for *MSH2* methylation was only present in *MSH2*-deficient tumors, we looked for germline mutations in 35 of 46 patients from which germline DNA was available for mutational analysis. Table 1 summarizes the clinical, genetic and epigenetic data from all 46 *MSH2*-deficient CRCs, and Figure 2A illustrates a representative example of absent *MSH2* expression in a MMR-deficient tumor. Eighty percent of patients (28 of 35) had a germline alteration in the *MSH2* gene. Among these, 24 cases had well-established pathogenic mutations, while the remaining 4 cases harbored unclassified variants in the *MSH2* gene: two patients with c.4G>A mutations, one with a c.942G>A mutation, and one individual had c.1316_1318delCTC. *In silico* analysis using the *Polyphen* prediction tool (<http://genetics.bwh.harvard.edu/pph/>) revealed c.4G>A mutation to be "possibly damaging", while c.942G>A mutation was considered "silent" (25). Consequently, of the 24 Lynch Syndrome patients with a confirmed germline mutation in

MSH2, 7 cases (29%) displayed the simultaneous presence of both a pathogenic germline mutation and somatic promoter methylation of the *MSH2* gene, suggesting *MSH2* promoter hypermethylation serves as a 'second hit' in these tumors.

***EpCAM* deletions in somatic *MSH2*-methylated CRCs**

Recent reports have proposed a role for deletion of the 3'-end of the *EpCAM* gene as a mechanism for *MSH2* methylation in Lynch Syndrome subjects who do not have germline mutations in *MSH2*. In our group of *MSH2*-deficient Lynch Syndrome CRCs, 7 of 11 cases with *MSH2* methylation also had simultaneous pathogenic *MSH2* germline mutations in the other allele, while the remaining 4 cases (patients C15, C17, C31 and C47, Table 1) either did not show pathogenic germline mutations, or we did not have sufficient materials to perform mutation analysis. In an attempt to understand the underlying cause for the somatic *MSH2* methylation in these tumors, we studied *EpCAM* deletions in these 11 *MSH2* methylation-positive patients. Three of the four patients without pathogenic germline *MSH2* mutations showed evidence for *EpCAM* deletion. This is consistent with the observations from previous studies that deletions in this gene are rare, and represent a mechanism for *MSH2* methylation in a small proportion of Lynch Syndrome patients that show 'germline' methylation in this MMR gene.

Overall, of the 35 *MSH2*-deficient Lynch syndrome cases which were analyzed for germline mutations in *MSH2* and *EpCAM* genes, 10 patients (29%) showed simultaneous presence of *MSH2* hypermethylation and mutation in either the *MSH2* or *EpCAM* genes, 19 cases (54%) had only germline *MSH2* mutations, while 6 subjects (17%) had neither germline mutations nor *MSH2* promoter methylation (Figure 2B). Since 70% (7 of 10) of patients with *MSH2* methylation also harbored germline mutations in this gene, this clearly suggest that methylation was the second inactivating event in these tumors.

***MSH2* methylated CRCs share features of CIMP CRCs**

Since frequent hypermethylation of many genes is one of the characteristic features of tumors with CIMP, we next determined associations between *MSH2* hypermethylation and various clinical, genetic and epigenetic factors. Since CIMP is present in a majority of sporadic MSI CRCs (due to *MLH1* methylation) and as many as 30–40% of sporadic MSS cancers(13), we also studied detailed associations between sporadic MSI or MSS subgroups of tumors and the *MSH2*-deficient Lynch Syndrome cases (Table 2 and 3). As expected, patients with sporadic MSI and MSS cancers were significantly older than the *MSH2*-deficient Lynch Syndrome cases. Sporadic MSI tumors were more frequent in females than males (sporadic MSI, 60%; *MSH2*-deficient Lynch Syndrome CRCs 33%; MSS, 35%). In addition, 92% of sporadic MSI and 60% of *MSH2*-deficient Lynch Syndrome CRCs were located in the proximal colon, in contrast to 30% of MSS cancers.

BRAF mutations were frequently present in sporadic MSI tumors (67%), but were seldom present in MSS tumors (5%), and did not occur at all in *MSH2*-deficient Lynch Syndrome CRCs (0%). However, *KRAS* mutations were never present in sporadic MSI cancers (0%), whereas 24% of *MSH2*-deficient Lynch Syndrome and 35% of MSS tumors harbored *KRAS* mutations.

We next investigated the methylation status of 6 CIMP-related loci (*MLH1*, *p16^{INK4a}*, *p14^{ARF}*, *MINT1*, *MINT2*, and *MINT31*; Figure 2C and Table 3) in all 268 CRCs, which included all MMR-deficient and -proficient CRCs. Not surprisingly, most sporadic MSI CRCs displayed a significant degree of methylation at all CIMP markers. Interestingly, we observed marked methylation at most CIMP-related markers in the *MSH2* methylated tumors, which was statistically significant at the *MINT1* and *MINT2* loci, when compared

with *MSH2* unmethylated cancers (Table 3). Of note, none of the *MSH2*-methylated cancers showed *MLH1* methylation, raising the possibility that inactivation of either MMR gene has similar functional consequences. For a better understanding of the role of *MSH2* methylation in the context of CIMP and sporadic MSI, we calculated the combined mean methylation scores based upon the number of CIMP-related markers methylated in each of the subgroups. As shown in Figure 2D, the mean methylation score was highest in sporadic MSI tumors (3.0; 95% confidence interval [CI], 1.9–4.1), followed by *MSH2*-methylated Lynch Syndrome tumors (2.4; 95% CI, 1.5–3.3), *MSH2*-unmethylated Lynch Syndrome tumors (1.1; 95% CI, 0.9–1.4), and was lowest in MSS tumors (0.7; 95% CI, 0.6–0.9). When we performed nonparametric multiple pairwise comparisons for the mean methylation scores in various subgroups of CRCs (Table 4), we noted that while the mean methylation scores were statistically different in each pairwise comparison, the scores were very similar when the results were compared between sporadic MSI and *MSH2*-methylated cancers. Collectively, the somatic *MSH2* methylation observed in Lynch Syndrome tumors indicates that *MSH2* is an important target of aberrant methylation, and that this is an important consideration in the pathogenesis of CRCs in Lynch Syndrome-*MSH2* type.

Discussion

Recent evidence for germline *MSH2* methylation in mutation-negative Lynch Syndrome patients with CRC prompted us to investigate whether *MSH2* may also be a target of somatic hypermethylation in the CRC tissues of patients with Lynch Syndrome-*MSH2* type. For this study, we analyzed a collection of 268 CRCs, which included 46 *MSH2*-deficient presumed Lynch Syndrome CRCs, 15 sporadic MSI CRCs, and 207 sporadic MSS CRCs. Prior to methylation analysis, we performed *MSH2* germline mutational analysis and noted that 80% of *MSH2*-deficient presumed Lynch patients harbored germline mutations in this gene (thus proving Lynch Syndrome-*MSH2* type). However, our subsequent analysis provides evidence that 24% (11/46) of *MSH2*-deficient Lynch Syndrome patients display *MSH2* hypermethylation in their tumor tissues. Moreover, 63% (7/11) of *MSH2* methylated CRCs had a simultaneous pathogenic germline *MSH2* mutation, suggesting that methylation may be the required second inactivating event in these tumors. No evidence for *MSH2* methylation was observed in normal tissues available for analysis or any of the sporadic CRCs, indicating that this epigenetic alteration occurs in a disease-specific manner. Additionally, while interrogating associations between *MSH2* methylation and methylation at multiple CIMP-related markers, we discovered that *MSH2*-methylated tumors also possessed markedly higher levels of promoter methylation, suggesting that the *MSH2* promoter may be a particular target of aberrant methylation in Lynch Syndrome CRCs.

Germline mutations in the DNA MMR genes *MLH1* and *MSH2* are the most frequent causes of Lynch Syndrome. However, in order for a tumor to arise in these individuals, the other, wild-type allele needs to be inactivated according to Knudson's "two-hit" hypothesis. This second hit can be a genetic alteration resulting in a deletion or somatic mutation (26,27), or can be an epigenetic alteration, a mechanism that has not been rigorously investigated in Lynch Syndrome tumors. Epigenetic inactivation of *MLH1* is the primary cause of sporadic MSI CRCs (which make up at least 12% of all CRCs), and germline epimutations in *MLH1* gene have been described in some Lynch Syndrome patients (16,17,19). Likewise, recent evidence indicates that the *MSH2* gene is another target of germline epimutations in some *MSH2* mutation-negative Lynch Syndrome individuals (21,28). However, it is not clear whether *MSH2* methylation is strictly a germline event, or whether it can occur on a somatic basis.

Almost a decade ago, the first efforts to study the epigenetic regulation of the *MSH2* gene in CRC produced negative results, and investigators failed to observe evidence for *MSH2*

methylation in a small cohort of sporadic primary CRCs(10). Since this report, no study has investigated *MSH2* methylation in a large group of colorectal tumors and normal colonic mucosal tissues. Similar to the previous report, our study did not find *MSH2* methylation in any sporadic MSI CRCs or normal colonic tissues. However, we found that 24% (11 of 46) of the *MSH2*-deficient Lynch Syndrome cases showed aberrant methylation in the *MSH2* promoter. Interestingly, of the 24 *MSH2*-deficient Lynch Syndrome tumors with a confirmed pathogenic germline mutation, 29% of the tumors had a simultaneous germline mutation and promoter methylation, suggesting that methylation is the “second hit”, fulfilling Knudson’s two-hit hypothesis in these *MSH2*-deficient Lynch Syndrome CRCs.

Our results suggest that the *MSH2*-methylation observed in our collection of Lynch Syndrome tumors is a somatic event which is present in ~30% of *MSH2*-deficient Lynch syndrome CRCs, and is distinct from the previous reports where it was shown to be a heritable germline *MSH2* epimutation (21). There are several logical explanations for this new paradigm. First, the evidence for *MSH2* methylation in our collection of Lynch Syndrome CRCs was primarily present in patients with germline mutations in *MSH2*, rather than in germline mutation negative cases as reported previously (20,21). The essential observation is that *MSH2* methylation co-existed with a germline mutation in 63% of our Lynch Syndrome-*MSH2* type CRCs. Second, in our collection of Lynch Syndrome patients with *MSH2* deficiency, we did not find this epigenetic defect in the matched normal mucosa of *MSH2*-methylated tumors, arguing against a germline defect in these individuals. Third, we detected *EpCAM* deletions in only 3 of 11 patients presenting with *MSH2* hypermethylation, which further supports the notion that *MSH2* hypermethylation in our patients occurred in a somatic manner, and through a different mechanism than what has been previously reported (20,21).

This concept derives further support from our data that sought associations between *MSH2* hypermethylation and aberrant methylation of six classical CIMP-related markers (*MLH1*, *p16^{INK4a}*, *p14^{ARF}*, *MINT1*, *MINT2*, and *MINT31*) in *MSH2*-deficient Lynch Syndrome CRCs and sporadic MSI tumors. In this regard, our collection of tumors showed lower frequency (7%) of MSI-positive tumors. Although the frequency of MSI CRCs in our study were somewhat lower compared to 12–15% rates reported in Caucasian populations, our results are in general agreement with lower MSI frequencies typically observed in the Japanese and Spanish populations. To our surprise, we found that the mean methylation scores at CIMP-related markers were higher in *MSH2*-methylated Lynch Syndrome tumors compared with unmethylated tumors. Pairwise comparison analysis further revealed that although slightly higher, there were no significant differences in mean methylation scores between sporadic MSI (i.e., *MLH1*-methylated) and *MSH2*-methylated (Lynch Syndrome) CRCs. These data underscore the contribution of aberrant methylation to the evolution of Lynch Syndrome adenomas (29) and CRCs (14). Of note, the high levels of methylation observed in sporadic MSI and *MSH2*-methylated Lynch syndrome tumors, where the *MLH1* and *MSH2* genes serve as targets of aberrant methylation respectively, indicate the functional significance of somatic epigenetic inactivation of these genes in the pathogenesis of two completely different subtypes of CRC.

Although these findings provide new insights into the molecular pathogenesis of Lynch Syndrome CRCs, there are limitations to the interpretation of our work that may require attention in future investigations. We have studied a reasonably large collection of *MSH2*-deficient Lynch Syndrome CRCs, however, studies with larger numbers of documented Lynch Syndrome-*MSH2* type are required to validate our results. Although our data suggest that somatic methylation of *MSH2* can provide the second hit in Lynch Syndrome CRCs, due to lack of adequate materials and technical limitations, we were unable to prove whether the germline mutations and methylation occurred on two separate alleles in the *MSH2* gene.

The timing of somatic *MSH2* methylation is unknown, and future studies of *MSH2*-deficient adenomas in patients with known germline mutations may reveal if it is an early event.

In conclusion, this study provides previously unrecognized evidence for relatively frequent aberrant methylation of the *MSH2* gene promoter in the CRCs of patients with Lynch Syndrome-*MSH2* type. More importantly, we discovered that the aberrant *MSH2* methylation in these tumors was not a germline event, but evolved in a somatic manner. Furthermore, similar to *MLH1* methylation in sporadic MSI and CIMP-positive CRCs, the existence of somatic *MSH2* methylation in some proportion of Lynch Syndrome CRCs that do not express *MSH2* protein may serve as a surrogate marker for aberrant methylation, and hereditary CRC. Considering the technical and scientific challenges in identifying novel germline mutations in the *MSH2* gene that might help explain *MSH2* deficiency in individuals without an identifiable germline mutation in that gene, our data suggest that the detection of *MSH2* methylation may be useful in properly identifying and classifying such individuals. Finally, since epigenetic events are potentially reversible, the early diagnosis of *MSH2* methylation in suspected Lynch Syndrome patients may have prognostic implications, a concept that mandates further exploration in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

| | |
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| MMR | DNA mismatch repair |
| CRC | colorectal cancer |
| CIMP | CpG island methylator phenotype |
| MSI | microsatellite instability |
| HNPCC | hereditary nonpolyposis colorectal cancer |

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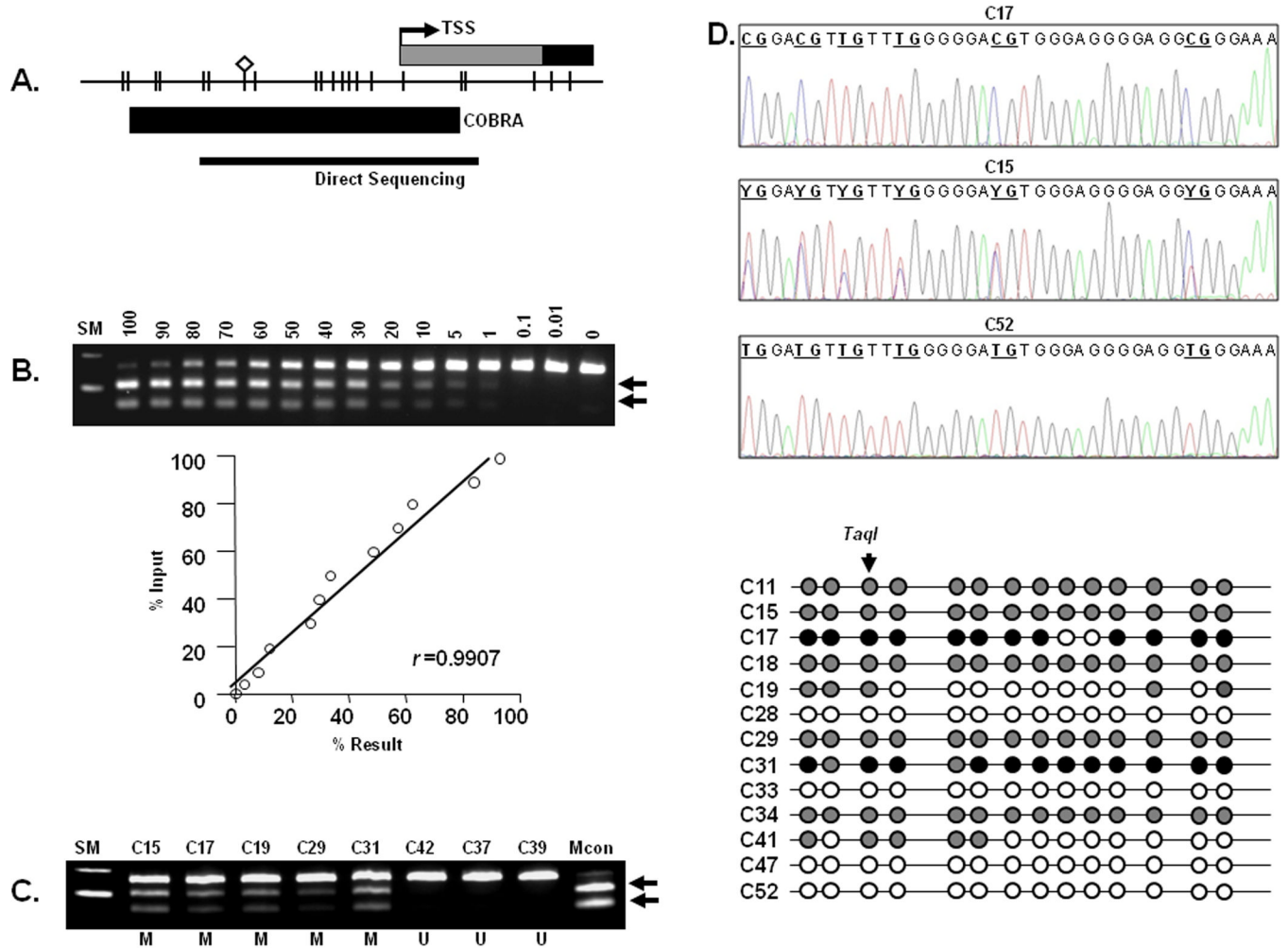


Figure 1.

A. Schematic depiction of the *MSH2* CpG island surrounding the transcription start site (TSS, black arrow): CpG dinucleotides are represented as short vertical lines. Location of the combined bisulfite restriction analysis (COBRA) amplicon and bisulfite genomic sequencing PCR products are indicated by thick and thin horizontal lines, respectively. The diamond represents the *TaqI* recognition site.

B. Determination of the quantitative accuracy of COBRA for the *MSH2* CpG island: As shown in the upper panel, COBRA was performed with the *TaqI* restriction endonuclease. Genomic DNA treated with *SssI* methylase (methylated) and normal control lymphocytes (unmethylated) were mixed at various ratios to determine the quantitative nature of the methylation assay. The numbers above each lane indicate the percentage of methylation. Arrows represent methylated alleles that are cleaved by *TaqI*; SM = size marker. The lower panel is an illustration of linear regression analysis for the methylation results shown in above panel: The calculated DNA methylation percentages are plotted as a function of the percentage input DNA treated with *SssI*. The correlation coefficient is 0.9907 for the *TaqI* digests, indicating that this is a robust assay.

C. Aberrant *MSH2* methylation in *MSH2*-deficient colorectal tumors: The numbers on top of each lane indicate various CRCs. Arrows indicate methylated alleles digested by *TaqI*. SM, size marker; Mcon, methylated control; M, methylated; U, unmethylated

D. Representative examples of MSH2 promoter bisulfite sequencing: The figures in the upper 3 panels illustrate representative examples of CRCs showing different degrees of methylation by direct bisulfite sequencing: case C17 from a methylated CRC shows methylated cytosines (indicated as CG) that remained cytosines following bisulfite conversion; case C15 with hemi-methylated CpGs (indicated as YG) showing overlapping C and T alleles; and case 52, from an unmethylated CRC showing CpG sites as TG as a result of bisulfite modification

The lower panel summarizes bisulfite sequencing results from 13 cases (9 methylated and 4 unmethylated) at all 14 CpG sites analyzed in the MSH2 promoter: White circles indicate unmethylated CpG sites (e.g. C28 and C33), gray circles indicate partially methylated CpG sites (e.g. C11 and C15) and black circles indicate fully methylated CpG sites (e.g. C17 and C31).

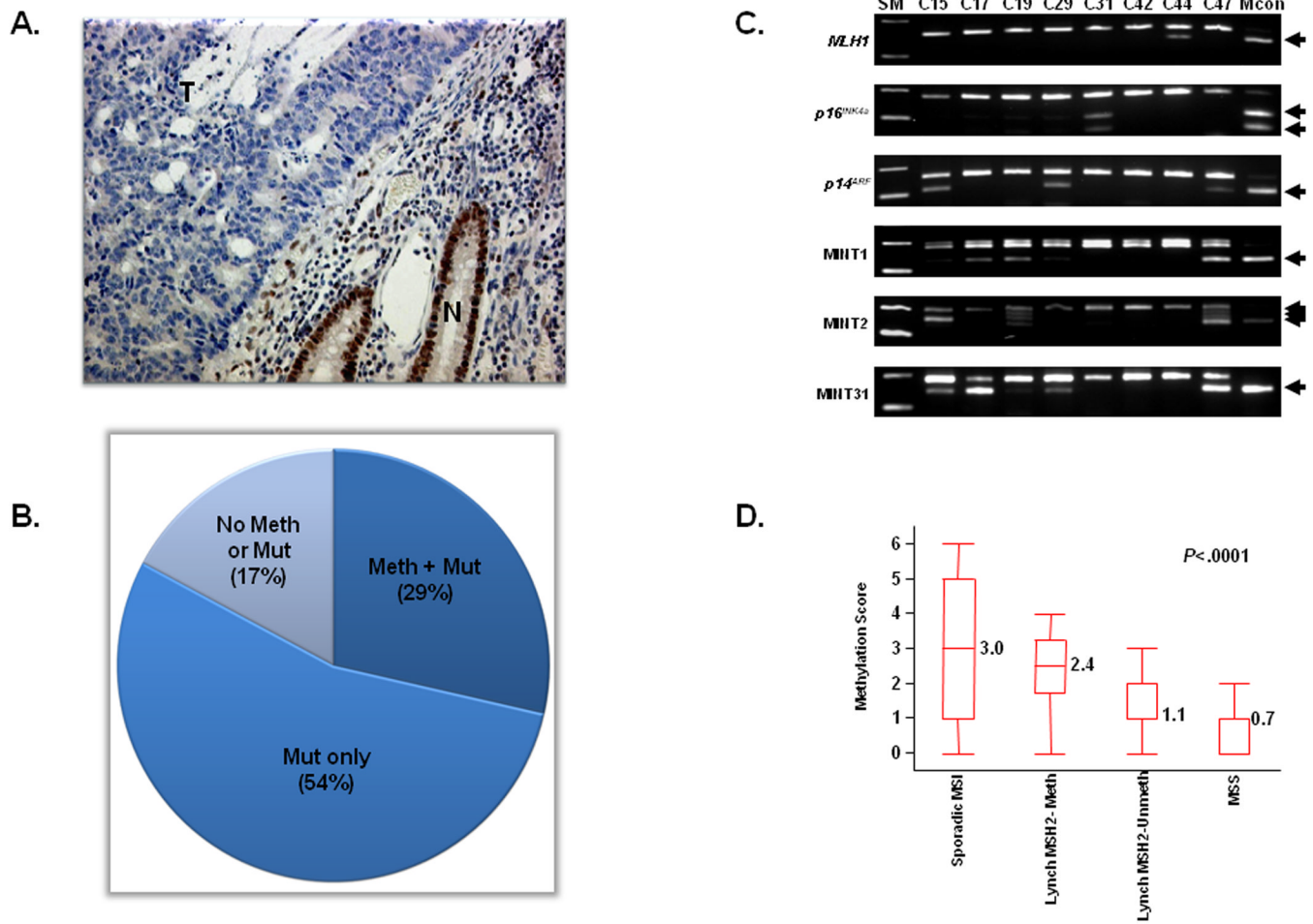


Figure 2.

A. *MSH2* immuno-histochemical staining: A representative example of a CRC with intact *MSH2* expression indicated by brown nuclear staining in the normal epithelial cells (N), while there is loss of *MSH2* expression in the tumor tissue (T) in the same patient.

B. Frequencies of germline mutations in *MSH2* and *EpCAM* genes and *MSH2* promoter methylation in *MSH2*-deficient CRCs: The pie chart illustrates data from 35 *MSH2*-deficient CRCs with a confirmed germline mutation in the *MSH2* or *EpCAM* genes or the presence of *MSH2* promoter methylation. Ten cases (29%) showed both germline mutations and promoter methylation, 19 cases (54%) harbored only germline mutations in *MSH2*, and 6 cases (17%) did not have either a germline mutations in either gene or promoter methylation of *MSH2*.

C. Representative examples of aberrant methylation in the 6 CIMP-related markers: The number in the top panel indicates various CRCs. Arrows indicate methylated alleles. SM, size marker; Mcon, methylated control

D. Mean methylation scores in various subgroups of CRCs: The mean methylation scores in each subset of CRCs were calculated by analyzing 6 CIMP-related loci (*MLH1*, *p16^{INK4a}*, *p14^{ARF}*, *MINT1*, *MINT2*, and *MINT31*). In the box-plot diagrams, the horizontal line within each box represents the median. The limits of each box are the inter-quartile ranges. The whiskers are the maximum and minimum values. The numbers next to each box denote the mean methylation score. The *P* values above the square panels were based on Kruskal-

Wallis 1-way analyses of variance on ranks. Statistical differences among any two individual groups are shown as pairwise comparisons in Table 4.

Summary of MSH2 expression, methylation, germline mutations and the associations with other clinical and genetic factors in 46 MSH2-deficient Lynch Syndrome CRCs

Table 1

| Case | Age | Location | MSH2 Protein expression | MSH2 Methylation | Germline MSH2 mutation ^a | Germline EpCAM mutation ^a | BRAF | KRAS |
|------|-----|----------|-------------------------|------------------|-------------------------------------|--------------------------------------|------|---------|
| C19 | 31 | Distal | — | + | c.840_841insT | — | none | c.35G>A |
| C11 | 40 | unknown | — | + | c.2291G>A | — | none | none |
| C52 | 52 | Proximal | — | + | c.2584G>T | — | none | c.38G>A |
| C18 | 31 | unknown | — | + | c.28C>T | — | none | c.38G>A |
| C29 | 50 | unknown | — | + | c.795delT | — | none | none |
| C41 | 42 | Proximal | — | + | c.2575G>T | — | none | none |
| C34 | 32 | unknown | — | + | deletion (exon 3–7) | n. a. | none | c.38G>A |
| C01 | 53 | unknown | — | — | c.1720C>T | n. a. | none | c.38G>A |
| C12 | 32 | unknown | — | — | c.1906G>C | n. a. | none | none |
| C13 | 67 | unknown | — | — | exon 12, 4bp deletion | n. a. | none | none |
| C14 | 39 | Distal | — | — | c.28C>T | n. a. | none | none |
| C22 | 43 | Proximal | — | — | c.973_974insT | n. a. | none | none |
| C25 | 42 | Distal | — | — | c.972_973ins184 | n. a. | none | c.38G>A |
| C27 | 62 | Proximal | — | — | c.416delA | n. a. | none | none |
| C03 | 58 | unknown | — | — | c.942+3A>T | n. a. | none | c.35G>A |
| C32 | 46 | unknown | — | — | c.942+3A>T | n. a. | none | none |
| C35 | 36 | Proximal | — | — | c.942+3A>T | n. a. | none | none |
| C36 | 46 | Proximal | — | — | c.2237_2240delTTCAT | n. a. | none | none |
| C37 | 47 | Distal | — | — | c.2584G>T | n. a. | none | none |
| C39 | 37 | Proximal | — | — | deletion (exon 1–2) | n. a. | none | none |
| C04 | 37 | unknown | — | — | c.2006-1G>C | n. a. | none | none |
| C42 | 40 | unknown | — | — | c.1835C>G | n. a. | none | none |
| C08 | 55 | unknown | — | — | c.1077-2A>G | n. a. | none | none |
| C09 | 26 | Distal | — | — | c.30_31insA | n. a. | none | c.38G>A |
| C15 | 28 | unknown | — | + | unclassified variant (c.4G>A) | + | none | none |
| C17 | 45 | unknown | — | + | unclassified variant (c.4G>A) | + | none | none |
| C26 | 39 | unknown | — | — | unclassified variant (c.942G>A) | n. a. | none | none |

| Case | Age | Location | MSH2 Protein expression | MSH2 Methylation | Germline MSH2 mutation ^a | Germline EpCAM mutation ^a | BRAF | KRAS |
|------|-----|----------|-------------------------|------------------|--|--------------------------------------|------|---------|
| C07 | 33 | unknown | — | — | unclassified variant (c.1316_1318delCTC) | n. a. | none | none |
| C31 | 60 | Proximal | — | + | none | + | none | none |
| C16 | 30 | unknown | — | — | none | n. a. | none | none |
| C02 | 61 | unknown | — | — | none | n. a. | none | none |
| C23 | 45 | Proximal | — | — | none | n. a. | none | none |
| C40 | 26 | unknown | — | — | none | n. a. | none | none |
| C43 | 35 | Distal | — | — | none | n. a. | none | none |
| C44 | 49 | Proximal | — | — | none | n. a. | none | none |
| C47 | 46 | Distal | — | + | n. a. | — | none | c.35G>A |
| C28 | 51 | Proximal | — | — | n. a. | n. a. | none | none |
| C30 | 35 | Proximal | — | — | n. a. | n. a. | none | none |
| C38 | 49 | Proximal | — | — | n. a. | n. a. | none | none |
| C45 | 44 | Proximal | — | — | n. a. | n. a. | none | none |
| C48 | 61 | Distal | — | — | n. a. | n. a. | none | c.38G>A |
| C49 | 45 | Distal | — | — | n. a. | n. a. | none | c.35G>A |
| C05 | 43 | unknown | — | — | n. a. | n. a. | none | none |
| C50 | 69 | Proximal | — | — | n. a. | n. a. | none | none |
| C51 | 65 | Distal | — | — | n. a. | n. a. | none | none |
| C06 | 21 | unknown | — | — | n. a. | n. a. | none | none |

^a n.a.=not analyzed.

Association between *MSH2* methylation and various clinical, genetic and epigenetic factors in *MSH2*-deficient Lynch syndrome CRCs

Table 2

| Clinical Factor | MSH2 Methylation %(n) | | P value |
|----------------------------|-----------------------|------------------------|---------|
| | Methylated (n=11) | Unmethylated (n=35) | |
| Age | | | |
| | ≥65 | 9 (3) | 0.3152* |
| | <65 | 91 (32) | |
| Gender | | | 0.2419* |
| | Female | 37 (13) | |
| | Male | 63 (22) | |
| Location | | | 1.0* |
| | Proximal | 60 (12) | |
| | Distal | 40 (8) | |
| | Missing data | (6) (15) | |
| Genetic Factor | | | 0.3496* |
| <i>MSH2/EpCAM</i> Mutation | Mutation | 100 (10) | |
| | Mutation | 76 (19) | |
| Status | No mutation | 0 (0) | |
| | No mutation | 24 (6) | |
| | Not analyzed | (1) (10) | |
| <i>BRAF/KRAS</i> | <i>BRAF</i> mutant | 0 (0) | 0.1001* |
| | <i>BRAF</i> mutant | 0 (0) | |
| Mutation | <i>KRAS</i> mutant | 45 (5) | |
| | <i>KRAS</i> mutant | 17 (6) | |
| Status | Both wild type | 55 (6) | |
| | Both wild type | 83 (29) | |
| Epigenetic Factor | | | 0.4176* |
| <i>MLH1</i> | Methylated | 0 (0) | |
| | Methylated | 6 (2) | |
| | Unmethylated | 100 (11) | |
| | Unmethylated | 94 (33) | |
| <i>p16^{INK4a}</i> | Methylated | 20 (2) | 0.1722* |
| | Methylated | 6 (2) | |
| | Unmethylated | 80 (0) | |
| | Unmethylated | 94 (32) | |
| <i>p14^{ARF}</i> | Methylated | 50 (5) | 0.0664* |
| | Methylated | 21 (7) | |
| | Unmethylated | 50 (5) | |
| | Unmethylated | 79 (27) | |
| <i>MINT1</i> † | Methylated | 60 (6) | 0.0493* |
| | Methylated | 26 (9) | |
| | Unmethylated | 40 (4) | |
| | Unmethylated | 74 (25) | |

| | MSH2 Methylation %(n) | | P value |
|---------------------|-----------------------|------------------------|----------|
| | Methylated (n=11) | Unmethylated (n=35) | |
| MINT2 [±] | Methylated | 6 (2) | <0.0001* |
| | Unmethylated | 94 (32) | |
| MINT31 [±] | Methylated | 47 (16) | 0.8700* |
| | Unmethylated | 53 (18) | |

* P values were calculated by the X² test.

Table 3
Frequency of DNA methylation at each epigenetic Marker in CRCs and its association with MSH2 expression and MSI Status

| | MSH2 Deficient CRC | | | MSH2 Proficient CRC | | P value |
|-------------------|--------------------------|------------------------|----------------|---------------------|-----------------------|---------|
| | Lynch Syndrome (n=46) | Sporadic MSI (n=15) | MSS (n=207) | | | |
| Clinical | | | | | | |
| Age | ≥65 | 7 (3) | 80 (12) | 51 (105) | <0.0001 ^a | |
| Factor | <65 | 93 (43) | 20 (3) | 49 (102) | <0.0001 ^b | |
| | | | | | 0.0283 ^c | |
| Gender | Female | 33 (15) | 60 (9) | 35 (73) | 0.0372 ^a | |
| | Male | 67 (31) | 40 (6) | 65 (134) | 0.864 ^b | |
| | | | | | 0.0553 ^c | |
| Location | Proximal | 60 (15) | 92 (12) | 30 (60) | 0.0597 ^a | |
| | Distal | 40 (10) | 8 (1) | 70 (143) | 0.0022 ^b | |
| | Missing data | (21) | (2) | (4) | <0.0001 ^c | |
| Genetic | KRAS/BRAF | 0 (0) | 67 (10) | 5 (10) | <0.0001 ^a | |
| Factor | Mutation | 24 (11) | 0 (0) | 35 (73) | 0.0724 ^b | |
| | Status | 76 (35) | 33 (5) | 60 (124) | <0.0001 ^c | |
| Epigenetic | MSH2 | 24 (11) | 0 (0) | 0 (0) | 0.0364 ^a | |
| Factor | Unmethylated | 76 (35) | 100 (15) | 100 (207) | <0.0001 ^b | |
| | | | | | 1.0 ^c | |
| | MLH1 | 4 (2) | 60 (9) | 0 (0) | <0.0001 ^a | |
| | Unmethylated | 96 (44) | 40 (6) | 100 (207) | 0.00259 ^b | |
| | | | | | <0.0001 ^c | |
| | p16 ^{INK4a} * | 9 (4) | 53 (8) | 16 (33) | 0.000237 ^a | |
| | Unmethylated | 91 (40) | 47 (7) | 84 (174) | 0.2444 ^b | |

| | MSH2 Deficient CRC | | MSH2 Proficient CRC | | P value |
|----------------------------|--------------------------|------------------------|------------------------|------------------------------|----------------------------|
| | Lynch Syndrome (n=46) | Sporadic MSI (n=15) | Sporadic MSI (n=15) | MSS (n=207) | |
| <i>p14^{ARF}</i> * | Methylated 27 (12) | 33 (5) | 8 (16) | 0.6545 ^a | 0.0003 ^c |
| | Unmethylated 73 (32) | 67 (10) | 92 (191) | 0.00018 ^b | 0.0011 ^c |
| MINT1 * | Methylated 34 (15) | 40 (6) | 9 (19) | 0.6798 ^a | |
| | Unmethylated 66 (29) | 60 (9) | 91 (188) | < 0.0001 ^b | 0.0003 ^c |
| MINT2 * | Methylated 18 (8) | 53 (8) | 20 (41) | 0.00818 ^a | |
| | Unmethylated 82 (36) | 47 (7) | 80 (166) | 0.8049 ^b | 0.0025 ^c |
| MINT31 * | Methylated 48 (21) | 60 (9) | 19 (40) | 0.4116 ^a | |
| | Unmethylated 52 (23) | 40 (6) | 81 (167) | < 0.0001 ^b | 0.0002 ^c |

All P values were calculated by the χ^2 test.

^a P values were calculated between MSH2 deficiency vs. sporadic MSI

^b P values were calculated between MSH2 deficiency vs. MSS cancers.

^c P values were calculated between sporadic MSI vs. MSS.

* Two cases were not analyzed in MSH2 deficiency cases.

Table 4

Pair-wise comparisons of methylation scores with between sporadic MSI and MSH2-deficient Lynch syndrome CRCs and *MSH2* methylation status

| | No. | Mean Methylation Score (95%CI) |
|--|-----|--------------------------------|
| Sporadic MSI | 15 | 3.00 (1.89–4.11) |
| Lynch Syndrome with MSH2 methylation | 10 | 2.40 (1.50–3.30) |
| Lynch Syndrome with MSH2 unmethylation | 34 | 1.12 (0.86–1.37) |
| MSS | 207 | 0.72 (0.58–0.86) |

| | Pairwise Comparison | <i>P</i> value* |
|--|---|-------------------|
| | Sporadic MSI vs. Lynch Syndrome with <i>MSH2</i> Methylation | 0.8684 |
| | Sporadic MSI vs. Lynch Syndrome with <i>MSH2</i> Unmethylation | 0.0044 |
| | Sporadic MSI vs. MSS | <0.0001 |
| | Lynch Syndrome with <i>MSH2</i> Methylation vs. Lynch Syndrome with <i>MSH2</i> Unmethylation | 0.0033 |
| | Lynch Syndrome with <i>MSH2</i> Methylation vs. MSS | 0.0001 |
| | Lynch Syndrome with <i>MSH2</i> Unmethylation vs. MSS | 0.0024 |

P values were based on Steel-Dwass test.