CHAPTER 8

Development of Sporadic Microsatellite Instability in Colorectal Tumors Requires Hypermethylation at Methylated-IN-Tumor Loci in Adenoma

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Abstract

Background: Microsatellite instability (MSI) and genomic hypermethylation of methylated-in-tumor (MINT) loci are known strong prognostic indicators of a subgroup of sporadic colorectal cancer (CRC) patients. A study was designed to determine whether methylation of MINT loci during adenoma progression to CRC is related to MSI in CRC.

Methodology: Methylation index (MI) was assessed by absolute quantitative assessment of methylated alleles (AQAMA) at seven MINT loci in CRC and contiguous adenomatous and normal tissues of 115 patients using in situ histopathology analysis. Results were then validated in primary tumor tissue from an independent group of 54 CRC patients.

Results: Increased methylation levels of MINT loci 1 and 31 were significantly associated with MSI and shown to be adenoma-specific. The total MI and number of methylated loci were 3-fold ($P=0.02$) and 5-fold ($P=0.004$) higher, respectively, in adenomas associated with microsatellite stable (MSS) versus MSI-High primary CRC. MINT methylation levels correlated with mismatch repair protein expression, MSI, B-RAF(V600E) mutation status, human mut-L homologue 1 (hMHL1) methylation status and CRC survival in an independent patient group.

Significance: Methylation levels of specific MINT loci were prognostic indicators of colorectal adenomas that will develop into sporadic microsatellite instable CRCs. The study indicates that increased MINT locus methylation precedes MSI in absence of malignant invasive tumor features and may have clinical pathology utility.
Introduction

Epigenetic changes in epithelial cells, such as DNA methylation of CpG islands, have been related to the genesis and progression of some gastrointestinal (GI) cancers\(^1,2\). Aberrations in DNA methylation are considered to be as important as genetic alterations in GI tumor initiation and progression. In colorectal cancer (CRC), both hypomethylation and hypermethylation of promoter-region related CpG islands have been correlated with clinical and pathology parameters\(^3,4\). A number of tumor-related gene promoter regions have been demonstrated to be methylated in early dysplastic stages of the colonic malignant pathway such as in hyperplastic polyps\(^5\), aberrant crypt foci\(^6,7\) and adenomas\(^8-10\). Progressive genomic and epigenomic aberrations may be linked in CRC\(^11\). For instance it has been reported that widely increased methylation in sporadic CRCs overlaps with microsatellite instability (MSI)\(^12-15\). Studies have been performed to pinpoint the onset of MSI in hereditary nonpolyposis colorectal cancers (HNPCC) that show germline mutations (mt) in mismatch repair (MMR) genes\(^13,14,16,17\), but as yet no study has examined this process in sporadic CRC. Further, the relation between genomic instability and epigenomic aberrations during sporadic CRC progression is not clear.

We recently described on-slide sodium bisulfite modification (SBM) technique for gene methylation analysis in small (1-2 mm\(^2\)) tissue areas isolated from a single section of paraffin-embedded archival tissue (PEAT)\(^18\). On-slide SBM allows comparison of gene methylation in the primary CRC and a contiguous adenoma lesion within the same tissue section. Genomic aberrations such as MSI and epigenomic changes can be tested at the same time from the same microdissected tumor lesion. Simultaneous assessment of CRC, precursor and normal tissues in the same PEAT section would provide valuable information that has not been available in the past.

In a previous study, our group assessed methylated-in-tumor (MINT) loci 1, 2, 12 and 31 and demonstrated technical feasibility of detecting methylation level differences between different colorectal tissue areas within the same tissue section using these loci\(^8\). MINT loci are conservative human genomic sequences that adhere to the CpG island definition,\(^19,20\) found in non-coding genomic regions, and gene regulatory or other functional attributes to date are unknown. We have previously shown the clinical utility of methylation levels of specific MINT loci in rectal cancer\(^20\) and melanoma\(^4\) and other groups have studied MINT locus methylation in colorectal\(^20,22\) and gastric cancers\(^23,24\). Methylation of MINT loci has also linked to MSI in CRC\(^25,26\); however this event has not been studied in adenomas. Therefore, we examined the methylation level of seven MINT markers in cancer, precancer, and normal tissue.

The objective was to identify if MINT locus hypermethylation influences MMR during early stages of CRC development. Methylation at MINT loci was quantified using the on-slide SBM technique combined with AQAMA. We further assessed MSI status, K-RAS mt at codons 12 and 13, B-RAF V600E mt, and methylation status of the MMR gene, \textit{MutL homolog 1 (hMLH1)}, in primary CRC as well as the contiguous precursor lesion in order to rigorously assess whether these events occur at early stages of CRC development. Our hypothesis was that adenoma tissue adjacent to MSI-high (MSI-H) invasive CRC tissue has increased MINT locus methylation levels, methylation of the \textit{hMLH1} promoter region, and...
MSI in comparison to precursor cancer lesions adjacent to microsatellite stable (MSS) cancers. We then validated in an independent patient group that MINT methylation levels positively correlated to \( hMLH1 \) methylation index (MI), MSI-status, B-RAF V600E mt, MMR protein expression, and disease survival.

**Materials and Methods**

**Patient Specimens**

For the first phase of the study, we searched the cancer registry database at Saint John’s Health Center (SJHC) to identify patients whose resected CRC specimen contained histopathologically confirmed areas of adenoma as well as invasive cancer. Excluded were any cases without available PEAT specimens. Consecutive patients were identified in reverse chronological order until an adequate sample size was reached. The final cohort of 115 patients underwent surgical resection of CRC between 1996 and 2009.

A single H&E section was prepared and mounted and 7-μm sections were consecutively cut and mounted on silane-coated glass slides for DNA studies. Areas of adenoma (serrated and non-serrated), carcinoma and normal tissue as well as the adenoma type were identified by a surgical oncology pathologist (R.R.T.) with expertise in CRC pathology.

Archived CRC primary tumor tissue PEAT blocks collected from a cohort of 54 patients that underwent surgical tumor resection at the Leiden University Medical Center (LUMC) between 1990 and 2001 were used for the validation study. All patients had been previously analyzed for MMR sufficiency at LUMC’s pathology department. From each PEAT block, a single section was cut for HE staining, and 7-μm sections were consecutively cut on coated slides for on-slide SBM. Tumor areas were identified and marked by an expert CRC pathologist (H.M.). Study protocols were approved by the institutional review boards at the LUMC and Saint John’s Health Center.

**AQAMA and \( hMLH1 \) Methylation Assessment**

DNA from PEAT was modified in situ by sodium bisulfite according to our previously reported protocol. AQAMA of MINT loci 1, 2, 3, 12, 17, 25 and 31 was performed and data analyzed as previously described. \( hMLH1 \) methylation status was analyzed by capillary-array-electrophoresis methylation-specific PCR (CAE-MSP). Primer sequences were those whose correlation with \( hMLH1 \) protein expression had been validated in previously reported studies.

**MSI, K-RAS and B-RAF mt Analysis**

For MSI assessment in the first phase study, an additional tissue section from each of the specimens was deparaffinized and stained with hematoxylin to identify tumor cells for DNA isolation, as previously described. Normal epithelial cells were harvested either from a separate tissue block of the same specimen (i.e. from uninvolved resection margins). As markers for MSI assessment, we used three quasi-monomorphic mononucleotide repeats BAT25, BAT26 and BAT40 and two microsatellite, dinucleotide repeats D2S123 and D5S346 according to the revised Bethesda guidelines. Forward primers (supplementary table S1) were
dye-labeled for automated high-throughput multiplex detection by CAE 37 (CEQ 8000XL, Beckman Coulter, Fullerton, CA). PCR product fragment length differences among different tissue categories were visualized by the CEQ software (Beckman Coulter). PCR products from the five amplified microsatellite regions in adenoma and cancer were compared to the reference normal epithelium. K-RAS (codons 12 and 13) and B-RAF (V600E) mt were assessed by a peptide nucleic acid (PNA) clamp– and locked nucleic acid (LNA) probe–based quantitative real-time PCR assay as previously described 38, 39. These assays were performed in triplicate and carried out at least twice to confirm accuracy. A gene in a specimen was considered mutated when results were uniformly positive in the triplicates under the optimal conditions. Respective normal and positive PEAT and cell lines controls were run in each assay.

Second Patient Group MMR and mt Status Analysis

The diagnostic techniques used by the LUMC’s pathology department for MSI status and MMR protein expression assessment were as described 40. Briefly, MSI-status was assessed by MSI Analysis System (Promega Corp.; five mononucleotide and two pentanucleotide repeats) 41. Immunostaining of MMR proteins was performed with anti-MLH1 (clone G168-728; 1:50; BD Biosciences, NJ) and anti-PMS2 (clone A16-4; 1:50; BD Biosciences). IHC staining was performed on 4-μm-thick, PEAT sections from tissue microarray (TMA). IHC staining patterns of these MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells, or the centers of lymphoid follicles as internal controls as previously published 42. TMAs contained three cores punched from each case’s primary tumor. Individual cores of the TMA were scored as either positive (showing nuclear staining in at least some tumor cells) or negative. Patients were considered positive if at least one TMA tissue core showed nuclear staining and negative otherwise. Cases in which both tumor and internal control stained negative were not included in the study. Cases were scored by two independent reviewers (H.M. and N.F.C.C. de M.) and in case of a tie, both reviewers reassessed the slides for consensus. K-RAS 43 and B-RAF 44 mt were detected by means of sequencing as previously described.

Biostatistical Analyses

Significance of changes in MI at individual MINT loci and total MI was evaluated with non-parametric tests for related and independent sample sets (Wilcoxon’s rank-sum test, Mann-
Whitney’s u-test). Fisher’s exact test (two-tailed) was used to assess significance of differences between \( hMLH1 \) methylation prevalence among groups.

Spearman’s rank correlation coefficient between quantitative MINT MI and \( hMLH1 \) MI was assessed as a non-parametric measure of correlation. Correlations with clinical parameters were tested with Pearson’s \( \chi^2 \)-square and in case of ordinal variables with Mann-Whitney’s or Kruskal-Wallis’ u-tests. Postoperative distant recurrence probability disease-free and overall survival over time were visualized with Kaplan-Meier plots and significance was assessed with the log-rank test. Cox’s regression models considered the following variables that were entered in a stepwise manner: age, N-stage, MSI status, tumor differentiation. \( P<0.05 \) (two-sided) was considered significant. SPSS (SPSS Inc., Chicago, IL) statistical software version 16.0.1 was used for all analyses.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total Patient Group (n=115)</th>
<th>Patients with Cancer Tissues (n=79)</th>
<th>Microsatellite Instable (n=9)</th>
<th>Microsatellite Stable (n=70)</th>
<th>( P )-value</th>
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</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
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<td>Male</td>
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<td>35 (44%)</td>
<td>3 (33%)</td>
<td>33 (47%)</td>
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<td>44 (56%)</td>
<td>6 (66%)</td>
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<td>Mean (SE)</td>
<td>76.1 (1.2)</td>
<td>75.0 (1.0)</td>
<td>81.4 (7.3)</td>
<td>76.1 (10.6)</td>
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<td>Cecum</td>
<td>24 (21%)</td>
<td>16 (20%)</td>
<td>3 (33%)</td>
<td>14 (20%)</td>
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<td>Colon Ascendens</td>
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<td>9 (11%)</td>
<td>4 (44%)</td>
<td>5 (7%)</td>
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<td>Hepatic Flexure</td>
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<td>12 (13%)</td>
<td>2 (22%)</td>
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<td>Sigmoid Colon</td>
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<td>8 (11%)</td>
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<tr>
<td>Rectosigmoid</td>
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<td>9 (13%)</td>
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<td>Rectum</td>
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<td>17 (22%)</td>
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<td>18 (26%)</td>
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<td><strong>Adenoma Type</strong></td>
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<td>Sessile Serrated</td>
<td>9 (8%)</td>
<td>5 (6%)</td>
<td>2 (22%)</td>
<td>3 (4%)</td>
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<td>Classic</td>
<td>106 (92%)</td>
<td>74 (94%)</td>
<td>7 (88%)</td>
<td>67 (96%)</td>
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<td>-Villoglandular</td>
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<td>8 (10%)</td>
<td>2 (22%)</td>
<td>6 (9%)</td>
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<tr>
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<td>10 (13%)</td>
<td>1 (11%)</td>
<td>9 (13%)</td>
<td></td>
</tr>
<tr>
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<td>73 (63%)</td>
<td>52 (66%)</td>
<td>4 (44%)</td>
<td>48 (68%)</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s exact test evaluating differences of MSI status in sessile vs classic adenoma in patients with adenoma as well as carcinoma available for analysis (n=72)

**Table 1:** Patient and tumor characteristics.
Results

MINT Locus Methylation Status and MSI during CRC Development

In the first phase of the study, we assessed operative specimens from 115 patients who underwent open resection of CRC whose specimen, according to the pathology report contained adenoma as well as cancer (see table 1 for patient characteristics). We first reviewed the newly cut sections for our study. Adenoma cases with high-grade dysplasia were not selected to obtain good separation between the adenoma and carcinoma category, and only carcinoma tissue with evidence of at least submucosal invasion was selected. Subsequently, 27 specimens were deleted. Of the remaining 88 specimens that contained low- or medium-grade adenomatous dysplasia, 79 also had invasive carcinoma tissue. 50 of these specimens had normal tissue present.

MSI status was analyzed using five established genomic markers in normal and cancer tissue of 79 patients. Nine patients (11%) showed instability in ≥4 biomarkers and were classified as MSI-H. Sixty-five patients (83%) did not show a shift in any of the markers, and five (6%) patients had a single dinucleotide repeat affected. These 70 patients with ≤1 aberrant MSI-marker were classified as MSS. The median age of MSI-H patients was 81.4 yrs (range 69-91). figures 1 and 2 show a representative PCR product analyses of AQAMA and CAE.

Figure 1: Representative examples of molecular analyses in normal (A), adenoma (B) and cancer (C) tissue. AQAMA (MINT31) real-time PCR results showing results in triplicate of exponential amplification of methylated (M) and unmethylated (U) dye probe signal. Y-axis represents signal intensity, X-axis represents PCR cycle number.
We compared MIs of the three tissue categories (normal, adenoma, cancer) for each individual MINT locus and divided the cases into MSI-H and MSS (figure 3). Methylation levels of MINT loci 1, 2, 3, 12 and 31 were significantly higher in CRC than in normal epithelium. Methylation levels of MINT loci 1, 2, 12 and 31 were significantly higher in MSI-H CRC compared to MSS CRC. Methylation levels of MINT loci 1 and 31 were significantly higher in adenomas contiguous to MSI-H CRC than in adenoma contiguous to MSS CRC. The total MI of tumor-specific, MSI-related MINT loci 1, 2, 12 and 31 was 4.5 times higher in adenomatous tissue paired to MSI-H tumors (P=0.02, figure 4). The average number of tumor-specific MSI-related MINT loci showing MI>0.1 was 1.5 (SD±1.4) in MSI-H related adenomas versus 0.3 (SD±0.6) in MSS related adenomas (P=0.004, figure 4). MINT17 MI was MSI-related in CRC but was also present in normal tissue. MSI status was significantly correlated to right-sided tumor location and not to serrated polyp type (table 1). MI was significantly higher in the serrated adenomas only at MINT1 and MINT31 (P=0.002 and P=0.02, respectively) compared to non-serrated adenomas. There were no significant MINT methylation level differences between carcinoma tissue contiguous to serrated adenomas versus carcinoma tissue contiguous to classic adenoma. Methylation levels of the two serrated/MSI-H adenoma were relatively low compared to the non-serrated/MSI-H adenoma.

From this we conclude that increased methylation levels of MINT loci 1, 2, 12, 17 and 31 are markers of MSI-H sporadic CRCs and except MINT 17 these are tumor-specific events. Increased methylation of MINT 1 and 31 were identified as adenoma/MSI-specific events and these loci are differentially methylated in sessile serrated adenomas compared to non-serrated adenomas. The MSI-H precursor lesions identified by MINT loci methylation analysis did not show any distinct histopathologic features. The total MIs and number of affected biomarkers of the cancer/MSI-specific MINT loci show significant differences and are indicators of colorectal adenomas that will become a microsatellite instable CRC.
Figure 3: Box plots summarizing MI values for normal, adenoma and cancer tissue separated into MSS and MSI-H tumors for each individual MINT locus.
hMLH1 methylation status and MSI during CRC development

Subsequently we determined whether the relation between MINT methylation levels and MSI in colorectal precursor lesions can be explained by a relation to methylation of the hMLH1 MMR gene. Therefore, hMLH1 methylation status was assessed in normal, adenomatous and cancer tissues of the nine MSI-H patients and in a control group of 13 MSS patients of which normal, adenomatous and cancer tissues were available of the first phase study. All nine MSI-H patients’ tumors showed methylation of hMLH1 compared to 2 of 13 of the MSS controls (P<0.001). Six of 9 MSI-H adenomas showed methylation of hMLH1 compared to 2 of 13 adenomas paired to the MSS controls (P=0.02). In 6 MSI-H patients with high tumor MI (≥0.88), instability of at least four MSI-biomarkers could be shown in only three of six (50%) of the associated adenomas. The paired adenoma tissue of these six patients did show increased MI at the MSI-specific MINT loci (≥0.56) compared to their paired normal epithelium. Together, MI of MINT1, 2, 12 and 31 and collateral methylation of hMLH1 constitute specific biomarkers of adenomatous colorectal tissue that shows MSI or will develop into a sporadic MSI-H CRC. The results further demonstrated that MINT locus methylation along with hMLH1 methylation precede MSI in the sequence of CRC development before histopathology signs of cancerous invasiveness are present.

B-RAF and K-RAS mt during CRC development

Studies in CRC have shown correlations between increased methylation of important tumor-related genes and mt of B-RAF (V600E) and K-RAS (codons 12 and 13), often in combination with MSI-H15, 19, 45, 46. The significance is not clearly understood, however it has been suggested that these events synergistically induce a high-risk phenotype that results in a clinically dis-

Figure 4: Box plots representing normal, adenoma and cancer tissue separated into MSS and MSI-H tumors. (A) Y-axis represent total MI of 4 MSI-related MINT loci (MINT1, 2, 12 and 31). (B) the Y-axis represents the number of MSI-related MINT loci with MI>0.1.
tinct subtype of CRC. We determined B-RAF V600E and K-RAS mt in the same group used for \textit{hMLH1} methylation status analysis (9 MSI-H cases and 13 MSS cases): the mt incidence was 46% and 18% for K-RAS and B-RAF, respectively. B-RAF mt was shown in 44% of the MSI-H carcinomas and in 63% of the contiguous adenomas. By comparison, none of the MSS adenomas and carcinomas had B-RAF mt (\(P=0.003\) and \(P=0.02\), respectively). K-RAS mt was more frequent in MSS adenomas and carcinomas (54% and 62% respectively), compared with MSI-H lesions (13% and 22%, respectively), although this did not reach statistical significance (\(P>0.09\)). These results were as expected based on previous reports. Total methylation levels at MINT loci 1, 2, 12 and 31 were significantly increased in B-RAF mt adenomas (\(P=0.03\)) as well as B-RAF mt carcinomas (\(P=0.002\)). None of the MINT loci were differentially methylated in K-RAS mt versus wild-type adenomas or carcinomas. This assessment demonstrated that genetic and epigenetic events synergize in the earliest phase of CRC development.

Quantitative Methylation and Mismatch Repair

The methylation levels of five MINT loci dispersed were related to methylation of \textit{hMLH1} and to MSI. These findings were validated using a second independent cohort of CRC patients whose \textit{hMLH1} expression was known. We first corroborated whether increased MINT MI linearly correlates to \textit{hMLH1} MI, and subsequently whether it affects MMR at the protein level. MIs at the MSI-associated MINT loci (1, 2, 12, 17, 31) and \textit{hMLH1} MI were measured in 54 CRC specimens that were previously analyzed for MMR sufficiency. Two outcome parameters were analyzed: total MI (defined by the sum of MIs of MINT loci 1, 2, 12, 17 and 31) and number of methylated MINT loci. The number of methylated MINT loci in a specimen was determined as the number of MINT loci (1, 2, 12, 17 and 31) that exceeded the MSI-H related MI cut-off. The average MI + 1SD of MSS CRC specimens from the first phase study determined this cut-off for MSI-related methylation. Correlation analysis showed that both outcome parameters were significantly associated with \textit{hMLH1} MI with a correlation coefficient of 0.43 (\(P=0.002\)) and 0.400 (\(P<0.001\)) for total MI and number of methylated MINT loci, respectively. Furthermore, in Table 2 the results for the association of \textit{hMLH1} protein expression in relation to MI are given. \textit{hMLH1} underexpression

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Number of MINT loci methylated} & \textbf{Number of Patients} & \textbf{Average Methylation Index (SD)} & \textbf{P} & \textbf{IHC non-expressing*} & \textbf{P} \\
\hline
0 & 32 & 0.14 (0.22) & 0.007 & 5 (16) & 0.002 \\
1 & 7 & 0.08 (0.10) & 2 (29) \\
2 & 4 & 0.01 (0.01) & 1 (25) \\
3 & 5 & 0.72 (0.34) & 1 (20) \\
4 & 2 & 0.42 (0.25) & 2 (100) \\
5 & 4 & 0.43 (0.37) & 4 (100) \\
\hline
\end{tabular}
\caption{Relation of Methylated Loci and MI to \textit{hMLH1} Methylation Status and Protein Expression}
\end{table}

* Confirmed by underexpression of PMS2
was corroborated by underexpression of its co-protein PMS2 in all cases. Examples of IHC and CEA-MSP results are given in figure 5. Subsequently all MMR protein deficient cases showed MSI by PCR analysis. Again we analyzed two outcome parameters: number of affected MINT loci and total MI. These results demonstrated a linear relation of quantitative MINT methylation to \textit{hMLH1} down regulation and subsequent MMR deficiency, and importance of both the number of loci involved as well as total MI.

\textbf{Figure 5:} Representative IHC staining of \textit{hMLH1} and PMS2 proteins. In \textbf{A} and \textbf{B} examples of \textit{hMLH1} and PMS2, respectively, expressing CRC with their corresponding CAE-MSP results for \textit{hMLH1} showing unmethylated signal only. In \textbf{C}, an example of a CRC showing methylation of the \textit{hMLH1} promoter region with corresponding absence of nuclear staining of \textit{hMLH1} which is confirmed by underexpression of PMS2.

B-RAF and K-RAS mt were also assessed in this patient group. B-RAF mt cases (n=11, 20\%) were significantly associated with MSI-H (\(P=0.003\)) and under expression of \textit{hMLH1} (\(P=0.003\)). K-RAS mt tumors (n=14, 26\%) did not show any significant associations with mismatch repair parameters. B-RAF mt tumors had significantly increased methylation at MINT1, 2, 12, 17 and 31 (\(P<0.001, P<0.001, P=0.001, P<0.001\) and \(P=0.003\), respectively). None of the B-RAF mt tumors had K-RAS mt. Methylation levels did not differ significantly between K-RAS mt tumors and K-RAS wild-type tumors.

Clinical outcome in our validation group was analyzed with respect to the number of MINT loci that showed MSI-related methylation. The result (\textbf{figure 6}) confirmed previous reports of a direct correlation between methylation levels recurrence-free survival.
Multivariate analysis showed that number of methylated, MSI-related MINT loci was an independent predictor of distant recurrence-free survival (HR 0.20, 95% CI 0.04 - 0.96, \( P = 0.02 \)), disease-free survival (HR 0.33, 95%CI 0.12 - 0.87, \( P = 0.04 \)) and overall survival (HR 0.38, 95%CI 0.14 - 1.00, \( P = 0.05 \)) along with nodal stage, and patient age.

**Discussion**

Epigenetic silencing of *hMLH1* has been identified as the causative mechanism for MSI in non-hereditary CRC by several groups\(^{13-15, 48}\). Our study adds important findings in genomic and epigenomic events at an early stage of CRC development. We could demonstrate that the relation between MSI and methylation is present at the premalignant stage in colorectal tissue without cancer histopathology. MI as well as the number of MINT loci methylated was significantly increased in MSI-H versus MSS CRC tumors and importantly, this was also found in their precursor lesions. In adenomas associated with MSI-H tumors there was subsequently significantly more hypermethylation of *hMLH1* detected. This suggests

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**Figure 6:** Kaplan Meier curves for distant recurrence probability stratified into patients 0, 1-2 or 3-5 MINT loci with MSI-related methylation.
that CRC precursor lesions with a higher MI at MINT loci may be more likely to have the hMLH1 MMR gene methylated as well. This has not been reported to date. We were able to demonstrate this by combining on-slide SBM and AQAMA techniques on PEAT which allowed accurate comparison of MIs between CRC tissues and contiguous adenoma tissues. Previous approaches reported were not capable of accurate analysis based on comparable synchronous histopathology and depended on comparison of specimens of different stages of CRC formation from different patients. The study’s results demonstrate significant changes in epigenetic and genetic markers at the origin of the development of a subset of CRC tumors.

The relation between degree of MINT methylation and MSI was further validated on the MMR protein level in a second independent patient group. MINT MI linearly correlated with hMLH1 MI, and subsequently resulted in underexpression of hMLH1 protein and MSI. This suggests that deregulation of a CRC’s MMR system causing sporadic MSI results from the increased likelihood of methylation of hMLH1 in a tumor with increased MIs at MINT loci. This demonstrated the level of DNA methylation to be an underlying mechanism of sporadic MSI and that this accumulation of DNA methylation affects the MMR system early during CRC development. We corroborated the validity of the quantitative MINT methylation AQAMA assay further by reproducing prognostic value which previous studies have shown in colorectal and gastric cancer.

Recently some studies on DNA hypermethylation in CRC include B-RAF and K-RAS mt analyses. B-RAF mt is more frequently found in sporadic CRCs showing MSI-H (approximately 50%), CIMP+ and hMLH1 methylation and are possibly associated with adverse disease survival. Specific single-nucleotide mt of K-RAS have predictive value in stage III/IV CRC, likely related to systemic treatment response. However, as yet no study has demonstrated a functional relation between MMR impairment and the reduced cell signaling properties of K-RAS or B-RAF mutant CRCs. Our results confirm the association of the V600E B-RAF mt to MINT locus hypermethylation and add that this ensemble of events initiates early in CRC development in non-serrated adenomas with only low or intermediate dysplasia.

Early identification of patients at risk for developing MSI-H phenotype sporadic cancers by AQAMA of MINT loci may be of clinicopathology utility. An approach that may be useful in the future would be to examine whether other polyps collected during subsequent colonoscopies from the same patient have similar DNA methylation results on repetitive samplings; this may identify a high-risk predisposition to CRC development. The polyp recurrence probability of patients with adenomas with high MINT locus methylation could be studied and the frequency of follow-up colonoscopies may be adjusted for such patients. Our results further show that MINT loci methylation levels can discriminate normal from adenomatous tissue. The MINT biomarkers could also be a part of a screening panel tested on fecal DNA to identify patients that should undergo colonoscopy. Not only MSI-H precursor lesions may be identified by MINT biomarkers 1, 2, 12 and 31 but also MSS lesions may be identified by MINT3 methylation. Furthermore, it is known that MSI-H cancers respond differentially to common chemotherapeutics. For instance, irinotecan is suggested to be more effective than 5-FU in these tumors. With the development of new targeted drugs, preventive treatment regimens may be indicated for high-risk patients with adenomas that
show high MINT locus methylation. Also there is increasing evidence for MSI-induced generation of novel tumor-specific carboxy-terminal frameshift peptides (FSPs) in MSI-H cancers\textsuperscript{57}. Patients who have polyps with high MINT locus methylation may become a stratified target patient group for vaccination trials of FSP-based approaches.

This is the first study to demonstrate a correlation between MSI and MINT hypermethylation in CRC precursor lesions, and this may be attributed to suppression of the MMR system by progressive epigenetic events. Therefore, the degree of genomic hypermethylation in CRC precursors is implicated as a potential causative factor of sporadic MSI CRC. Specific MINT locus methylation identification may have utility in early identification of colorectal polyps that will develop into sporadic MSI-H cancers.
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