Quantitative Analysis of Methylation of Genomic Loci in Early-Stage Rectal Cancer Predicts Distant Recurrence

Michiel F.G. de Maat, Cornelis J.H. van de Velde, Martijn P.J. van der Werff, Hein Putten, Naoyuki Umetani, Elma Meershoek Klein-Kranenbarg, Roderick R. Turner, J. Han J.M. van Krieken, Anton Bilchik, Rob A.E.M. Tollaen, and Dave S.B. Hoon

ABSTRACT

Purpose
There are no accurate prognostic biomarkers specific for rectal cancer. Epigenetic aberrations, in the form of DNA methylation, accumulate early during rectal tumor formation. In a preliminary study, we investigated absolute quantitative methylation changes associated with tumor progression of rectal tissue at multiple genomic methylated-in-tumor (MINT) loci sequences. We then explored in a different clinical patient group whether these epigenetic changes could be correlated with clinical outcome.

Patients and Methods
Absolute quantitative assessment of methylated alleles was used to assay methylation changes at MINT 1, 2, 3, 12, 17, 25, and 31 in sets of normal, adenomatous, and malignant tissues from 46 patients with rectal cancer. Methylation levels of these biomarkers were then assessed in operative specimens of 251 patients who underwent total mesorectal excision (TME) without neoadjuvant radiotherapy in a multicenter clinical trial.

Results
Methylation at MINT 2, 3, and 31 increased 11-fold (P = .005), 15-fold (P < .001), and two-fold (P = .02), respectively, during adenomatous transformation in normal rectal epithelium. Unsupervised grouping analyses of quantitative MINT methylation data of TME trial patients demonstrated two prognostic subclasses. In multivariate analysis of node-negative patients, this subclassification was the only predictor for distant recurrence (hazard ratio [HR], 4.17; 95% CI, 1.72 to 10.10; P = .002), cancer-specific survival (HR, 3.74; 95% CI, 1.4 to 9.43; P = .003), and overall survival (HR, 2.68; 95% CI, 1.41 to 5.11; P = .005).

Conclusion
Methylation levels of specific MINT loci can be used as prognostic variables in patients with American Joint Committee on Cancer stage I and II rectal cancer. Quantitative epigenetic classification of rectal cancer merits evaluation as a stratification factor for adjuvant treatment in early disease.

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INTRODUCTION

Rectal cancer is the second most common cancer of the digestive system in the United States.1 Neoadjuvant therapy has improved local control of rectal cancer in patients undergoing total mesorectal excision (TME),2,4 but distant recurrence remains the major cause of disease mortality. Although tumor status of regional nodes is the most important predictor of metastasis, 20% of node-negative patients will have a recurrence at distant sites. This suggests that even early stages of tumors have potential for systemic metastasis and, therefore, molecular subclassification may be clinically relevant. Development of prognostic molecular biomarkers for rectal cancer would improve management and potential treatment stratification. Colon and rectal cancers are often assessed together in the analysis of molecular/genetic biomarkers. This is often due to the limited availability of rectal tumor for analysis, or rectal specimens are not procured from a specific clinical trial. We now know both colon and rectal cancers are different in etiology and treatment, as well as (epi)genetics.5 In this study, we have focused specifically on epigenetic changes of rectal cancers from a clinical trial.

Epigenetic instability, such as changes in genomic DNA methylation status, is an early event during GI tumor development and encompasses both hyper- and hypomethylation changes.6-8 Most epigenetic cancer studies focus on specific genomic
loci and analyze methylation status in a dichotomous manner, categorizing specimens as methylated or unmethylated. Also, in the majority of the studies assessing epigenetic changes and association with clinical outcome, nonquantitative measures are used, using a binary methylation status result. Absolute quantitative interpretation of methylation data would improve analysis of epigenetic events.9 Recently, we developed an assay for absolute quantitative assessment of methylated alleles (AQAMA) and showed quantitative methylation events to be associated with colorectal tumor progression.10 AQAMA measures the amount of methylated and unmethylated copy numbers simultaneously in a single reaction. The assay has excellent linearity in assessing DNA methylation levels and can be used on paraffin-embedded archival tissue (PEAT) sections treated with the on-slide (in situ) sodium bisulfite modification (SBM) technique that allows microdissected histology-oriented assessment of small (1 to 2 mm²) lesions.11,12 This allows efficient comparison of precursor adenoma and normal cells adjacent to tumor cells.

Methylation levels of methylated-in-tumor (MINT) loci have not been specifically tested for prognostic utility in rectal cancer. MINT loci are CpG dinucleotide–rich regions located in nonprotein-encoding DNA regions, and have been reported to become methylated in a tumor- and adenoma-specific manner in gastric and colon cancer.13-17 In a preliminary study, we quantified methylation levels of seven MINT loci at different stages of rectal tumor formation comparing paired normal-adenoma and adenoma-cancer tissues, and subsequently analyzed whether methylation level changes related to rectal tumor progression. Our developed hypothesis was that methylation levels at MINT loci have prognostic significance for early rectal cancer progression. We then assessed the potential prognostic utility of MINT loci in primary tumor tissues from patients enrolled in a multicenter, randomized, surgical clinical trial. In this translational study analysis, unsupervised cluster analysis identified a subclass of patients whose quantitative methylation data were independently prognostic of progression to distant disease.

PATIENTS AND METHODS

Tissue Specimens

In the preliminary study, patients who underwent surgery for rectal cancer with histopathologic-confirmed adenocarcinoma were identified from the cancer registry database at Saint John’s Health Center (Santa Monica, CA). Only patients who underwent surgery after 1995 were evaluated because of possible DNA degradation. Further selection of specimens was based on pathology-documented presence of tumor, as well as adenoma cells on the same tissue section.

For the clinical correlation studies, primary tumor PEAT specimens were obtained from 322 nonirradiated patients enrolled onto the multicenter, randomized, quality-controlled TME trial coordinated by the Dutch Colorectal Cancer Group.3 The trial investigated whether neoadjuvant radiotherapy (5 × 5 Gy) before TME improved local control compared with TME surgery alone in patients with all stages of rectal cancer. Trial eligibility criteria and follow-up protocols have been described previously.3,18,19 All TME trial patients enrolled at the Dutch multicenter study sites were eligible, further adhering to the following criteria: nonirradiated, TNM stage I-III, with no evidence of disease after surgery. We opted to analyze the nontreatment arm because potential effects of radiation on genomic methylation are not known. Research protocols for the methylation studies on PEAT were approved by the internal review boards of Saint John’s Health Center, John Wayne Cancer Institute (Santa Monica, CA), and Leiden University Medical Center (Leiden, the Netherlands).

DNA Preparation From PEAT Specimens for Preliminary and Clinical Studies

From the preliminary study specimens, two consecutive sections (4 and 7 µm) of each PEAT block were cut and placed on adhesive-coated slides. The 4 µm section was stained with hematoxylin and eosin (H&E) and mounted. Tissue areas with normal epithelial, classic adenomatous, and invasive cancer cells were identified and marked by an expert surgical pathologist (R.R.T.). The tissue categories were identified by histopathology. Cancer cells were only taken from areas with nuclear atypia and signs of invasion of tissue architectural boundaries, the hallmark of cancer. Adenomatous cells were only taken from areas with classic villous and/or tubular adenomatous dysplasia. We did not include adenomatous tissue in the analysis with highly dysplastic features without signs of invasion. The 7-µm section was treated by on-slide SBM as described previously.10 Target tissue areas were identified and microdissected under a light microscope. Isolated cells were digested and 1 µL of the lyase was used for polymerase chain reaction.

From the clinical study of TME trial patient specimens, tissue sections (7 µm) were cut from PEAT specimens and mounted on nonadhesive glass slides. Tumor-representative areas on H&E-stained sections were marked by a surgicalopathologist specializing in rectal cancer (J.H.J.M.v.K.). Two sections per patient were deparaffinized, and the marked tissue was carefully microdissected. DNA was isolated and modified by sodium bisulfite, as previously described.20 Salmon sperm DNA was added as a carrier.21 Double-stranded DNA and single-stranded DNA were quantified before and after SBM by PicoGreen and OliGreen assays (Molecular Probes; Invitrogen, Carlsbad, CA), respectively. Sufficient input DNA for AQAMA was determined as described.20 A salmon sperm DNA sample without tumor DNA was included in triplicate to assess background signal in all assays. Tissue blocks and isolated DNA were coded to prevent any bias.

AQAMA MINT Locus Methylation Level Assessment

Absolute quantitative assessment of methylated alleles at MINT loci 1, 2, 12, and 31 has been described previously.10 Unpublished primer and probe sets for the remaining three MINT loci were: MINT3, 5’-TGATGGTGTTATGTGATTTTGTTGTTTTGTTTGTT-3’ (forward), 5’-ACCTACACCCCTCACAACACG-3’ (reverse); MINT2, 5’-ACCTACGAAGGACAGA-3’ (methylated probe), 5’-TACCTACAAACAAAC-3’ (unmethylated probe); MINT7, 5’-ACCTACGAAGGACAGA-3’ (methylated probe), 5’-TTTGATGATGACTGGG-3’ (methylated probe), 5’-TTTGGATGATGACTGGG-3’ (unmethylated probe); and MINT25, 5’-GGGGAATGGGAGAATGTTGAAGGAGAATGTTGAAGGAGAATGTTGAAGGAGAATGTT-3’ (forward), 5’-ACCTACACCCCTCACAACACG-3’ (reverse), 5’-TTTGATGATGACTGGG-3’ (methylated probe), 5’-TTTGGATGATGACTGGG-3’ (methylated probe), 5’-TTTGGATGATGACTGGG-3’ (unmethylated probe), DNA samples were run in 384-well microplates in triplicate, and each plate contained individual marker cDNA standards with known copy numbers, allowing assessment of absolute methylated and unmethylated copy number. Controls for specificity of AQAMA for methylated and unmethylated sequences, as well as controls for nonspecific amplification, were included.20,22 Final analysis outcome was the methylation index (MI), calculated as: [copy number methylated alleles/copy number methylated alleles + copy number unmethylated alleles].

Profiling by Unsupervised Random Forest Clustering

For identification of patient clusters with similar MINT locus methylation profiles, we employed unsupervised random forest (RF) clustering.23 RF has been successfully applied in comparable data sets (Appendix 1, online only).24,25

RESULTS

MINT Locus Methylation Levels During Rectal Cancer Development

Sets of normal, adenomatous, and malignant PEAT tissues from 46 patients with rectal cancer were examined by AQAMA of MINT loci known to be differentially methylated in colorectal cancer.11 The H&E-stained sections cut from the tissue blocks that, according to the
Fig 1. Scatterplots of measured methylation indices in normal rectal epithelium, rectal adenoma tissue, and rectal cancer tissue for the 7 MINT loci studied. MINT, methylated in tumor; ns, not significant.
diagnostic pathology report, contained adenoma as well as cancer tissue, were evaluated histopathologically by an expert pathologist (R.R.T). In the 46 tissue sections, 19, 46, and 35 areas of normal epithelium, adenoma, and cancer tissue, respectively, were identified. This resulted into paired analyses of 19 normal-adenoma sets and 35 adenoma-cancer sets. Figure 1 shows scatterplots of the MI values in the three histopathology categories for each MINT locus. MINT loci 2, 3, and 31 underwent a significant increase in absolute mean methylation level during adenomatous transformation. There were no significant MINT methylation changes for any MINT locus during progression from adenoma to cancer. Subsequently, the significant increases were early events associated with dysplastic change of normal rectal epithelium. Because three MINT loci (2, 3, and 31) showed significant increase in methylation levels and the normal distribution of the quantitative methylation data sets in healthy rectal epithelium changes to non-normal in adenoma in four other loci (1, 12, 17, and 25; Appendix 2 and Table A1, online only), all seven MINT loci were considered to have potential utility to identify epigenetic subclasses in the clinical study patient group.

Sample Size Calculations
To establish the sample size for the clinical study, we performed power calculations using methylation results of the preliminary study and recurrence rates of the TME trial. It was calculated that 250 patients were sufficient to obtain significance for predicting distant recurrence with an \( \alpha \) level of .05% and 90% power. Because the available patient specimens from the trial were primary tumor PEAT blocks from various hospital sites, we allowed for 30% loss of patient...
samples due to availability and quality of tissue and DNA. We therefore required 75 additional patient samples, and the final sample size was set at 325 patient samples. Six hundred seventy-two patients fulfilled our study criteria (see Patients and Methods). Finally, DNA was isolated in 314 patient samples (in 11 patient samples, tumor cell number was insufficient). Subsequently, after processing and bisulfite treatment, only 251 of the 314 DNA isolations had sufficient input DNA for AQAMA. Characteristics of the 251 patients in the final analysis were not significantly different in prognostic factors and characteristics from the original trial population (Appendix Table A2, online only).

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<th>Table 1. Variable Importance by Gini Index and Comparison of Mean MINT Locus Methylation Index Values Between Identified Clusters</th>
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<td>MINT Locus</td>
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Abbreviation: MINT, methylated in tumor.
*Calculated by Mann-Whitney’s U test.

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<th>Table 2. Comparison of Clinical and Tumor Pathology Factors and MINT Locus Clusters</th>
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<td>Clinical and Tumor Pathology Factors</td>
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<td>N1 (1-3 positive)</td>
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<td>N2 (≥ 4 positive)</td>
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Abbreviation: MINT, methylated in tumor.
**MINT Locus Methylation Profile Identification**

To investigate whether rectal cancer can be grouped by methylation level at specific MINT loci, we performed unsupervised RF clustering on the quantitative methylation level results of patients from the TME trial. As an outcome, a multidimensional scaling (MDS) plot indicated the mutual distance between the samples based on methylation level of all seven MINT loci (Fig 2A). Inspection of the MDS plot indicated two groups of rectal cancer cases. To identify which patients belonged to which group, we performed an expectation maximization algorithm with a mixture of Gaussians (EM-MoG) analysis based on the Gaussian shape of patient clusters (Figs 2B and 2C). The EM-MoG algorithm allocated the patients based on the likelihood that they would fall under the normal (Gaussian) distribution of one of the two clusters. Subsequently, variable importance and the methylation patterns matching the identified clusters were analyzed (Fig 2D; Table 1). The 89 patients (35%) allocated to cluster 1 had significantly increased methylation at MINT3 and significantly decreased methylation at MINT1, 12, and 17 compared with patients in cluster 2. The unsupervised clustering results showed that subclasses of rectal cancers could be identified by differences in DNA methylation level of tested MINT loci. The Gini index indicated that MINT3 and MINT17 were the most important variables in forming the clusters.

**Clinicopathologic Correlation and Distant Recurrence Analyses**

There were no significant associations observed in epigenetic subclasses of rectal cancer to any of the investigated standard clinical or tumor-pathological factors (Table 2). The preliminary results demonstrated that methylation level differences at the specific MINT loci develop early during tumor formation. There was no significant relation between cluster allocation and clinicopathologic factors in node-negative tumors (Table 2). Because identification of stage I and II patients at risk for distant metastasis is clinically highly relevant and there was no dependence of the identified patient clusters to nodal status, we excluded stage III patients from distant disease recurrence analyses. We assessed the probability of distant disease recurrence, cancer-specific, and overall survival (OS). Because EM-MoG analysis is a probability-based cluster assignment algorithm, we performed multiple imputation analysis to correct for cases that have a small difference in probability to be assigned to either one of the clusters. In node-negative patients, cluster 1 patients had significant increased risk for distant recurrence ($P = .01$), shorter cancer-specific survival ($P = .02$), and shorter OS ($P = .05$; Figs 3A to 3C). At the time of the analyses, median duration of follow-up was 7.1 years (range, 2.5 to 9.8 years).

**Multivariate Analyses**

Multivariate analyses were performed to assess whether the observed prognostic value of the clusters was independent from standard prognostic variables for the complete patient group and for node-positive and negative patients (Table 3). T stage, N stage, circumferential margin status, distance of the tumor to the anal verge, and tumor differentiation were considered in a Cox regression analysis. In node-negative patients, the quantitative MINT locus methylation profile—of all the considered variables—was the only selected predictive factor for distant disease recurrence and cancer-specific survival. OS was also affected by T-stage in patients without nodal involvement.
Circumferential margin involvement of the tumor and short (<5 cm) distance of the tumor from the anal verge increased the risk of distant recurrence, and decreased cancer-specific survival and OS in node-positive rectal cancer patients. Possible dependence of the results on any of the 42 different study sites was evaluated in the published clinical trial report and was also ruled out (data not shown) in our analyses. The multivariate results show that the identified subclass of rectal cancers is independently predictive of distant recurrence in node-negative patients.

**MINT3 and MINT17**

The Gini index, indicating variable importance in RF clustering as shown in Table 1, demonstrated MINT3 and MINT17 to hold the most information to form the two clusters compared with the other five MINT loci. We continued to assess whether methylation levels at MINT3 and MINT17 have prognostic value as a separate marker set. The quantitative methylation data of MINT3 and MINT17 have prognostic value as a separate marker set.

![Figure 4A](https://example.com/figure4a.png)

**Figure 4A.** Four clearly separate clusters are formed and the corresponding methylation level differences between the clusters are plotted into the RF algorithm and the resulting MDS plot is shown in Figure 4B. Cluster 3, with 67 patients (27%), corresponds to the MINT locus profile (cluster 1) with a methylation index that is relatively low. In Kaplan-Meier analysis, cluster 3 patients are demonstrated to be at significantly increased risk for distant metastasis in node-negative patients compared with the other three clusters (Fig 4C). In multivariate analysis, the results showed that the high-risk cluster 3 was selected as the only independent factor among the variables analyzed that in node-negative patients predicted distant recurrence probability (hazard ratio [HR], 2.84; 95% CI, 1.22 to 6.62; \(P = .02\)), cancer-specific survival (HR, 3.29; 95% CI, 1.33 to 8.12; \(P = .01\)), and OS (HR, 2.21; 95% CI, 1.13 to 4.29; \(P = .02\)). It was concluded that patients at increased risk for distant metastasis can be defined as having tumors with a MINT3 methylation level more than 0.72 and MINT17 methylation level less than 0.14. The analysis also demonstrated that the specific combination of increased methylation at MINT3 and decreased methylation at MINT17 is required for the prognostic information.

**DISCUSSION**

Most studies of biomarkers in large bowel adenocarcinoma include both the colon and the rectum, even though rectal and colon cancers are treated differently. Moreover, right-sided and left-sided bowel adenocarcinomas have different molecular patterns; microsatellite instability and methylator phenotype are rarely seen in the rectum. Our data represent one of the largest clinical analyses of methylation biomarkers in rectal cancer specifically, and to our knowledge also demonstrate the first quantitative correlation between MINT methylation levels and disease progression.

The preliminary study demonstrated a progressive increase in methylation levels of specific MINT loci comparing normal and adenomatous rectal tissue. No significant change in methylation level at any MINT locus was detected comparing adenomatous and malignant rectal tissue. A correlation between methylation of MINT loci and development of adenomatous dysplasia has been reported. Our data are unique, as we used paired normal-adenoma cancer specimens, quantitative techniques, and analyzed rectal cancers only. The results of our clinical study identified two prognostic categories of...
rectal cancer disease recurrence and disease survival. The role of non-coding regions has been of much interest in that it may be influential in gene encoding regions.\textsuperscript{28-30} Especially interesting is that the chromosomal location (1p36) of the MINT3 locus, which undergoes methylation in most rectal adenomas, contains many cancer-related genes. Methylation of MINT loci 1, 2, 12, and 31 is often studied in relation to the CpG island methylator phenotype (CIMP) that forms a subclass of right colon tumors closely associated with microsatellite instability.\textsuperscript{31}

In our study, the unsupervised clustering analyses did not identify a CIMP associated with hypermethylation in the selected MINT loci (data not shown). Interestingly, a combination of relative hyper-as well as hypomethylation was observed in the identified subclasses. This specific combination was even required to show prognostic value on rectal cancer distant recurrence rates. This corroborates that CIMP does not occur in the rectum and that rectal cancer may have different epigenetic pathologic changes compared with proximal colon adenocarcinoma. Reported correlations between MINT 1, 2, 12, and 31 and clinicopathologic features overlap with the features of microsatellite instability (positive) tumors (right-sidedness, poor differentiation, early stage) and therefore our results cannot be compared.\textsuperscript{27,32,33} We previously showed relevance of the AQAMA technique testing methylation levels at MINT 1, 2, 12, and 31 and increased methylation at this loci detected by the AQAMA assay was significantly correlated to right-sided colon tumors.\textsuperscript{10}

Our preliminary study data indicate that methylation events at the measured MINT loci are related to early dysplastic proliferation of subclasses of rectal premalignancies, and MINT loci may be a clinical biomarker. Subsequently, in a large rectal cancer patient group, RF clustering was able to identify—in an unbiased manner—two groups of rectal cancer patients that were naturally present within the quantitative methylation data. This demonstrated that subclassification of rectal cancer patients can be made based on absolute quantitative methylation level differences.

There was no correlation between MINT methylation profile and nodal status; in node-negative patients, the MINT profile was the only selected variable in multivariate analyses for distant recurrence probability and, subsequently, for cancer-specific survival. Identifying stage I and II patients at risk for distant disease recurrence and assessing primary tumors for predictive genomic biomarkers would be important for stratifying adjuvant treatment. Moreover, given that accurate upstaging from stage II to III remains a difficult task,\textsuperscript{34} we approached this by a quantitative analysis of a specific panel of epigenetic biomarkers. The advantage of using genomic analysis is the stability of DNA compared with mRNA in PEAT, where, in the latter, does not occur in the rectum and that rectal cancer may have different epigenetic pathologic changes compared with proximal colon adenocarcinoma. Reported correlations between MINT 1, 2, 12, and 31 and clinicopathologic features overlap with the features of microsatellite instability (positive) tumors (right-sidedness, poor differentiation, early stage) and therefore our results cannot be compared.\textsuperscript{27,32,33} We previously showed relevance of the AQAMA technique testing methylation levels at MINT 1, 2, 12, and 31 and increased methylation at this loci detected by the AQAMA assay was significantly correlated to right-sided colon tumors.\textsuperscript{10}

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**AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**

The author(s) indicated no potential conflicts of interest.

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**Provision of study materials or patients:** Cornelis J.H. van de Velde, Roderick R. Turner, Anton Bilchik

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**Final approval of manuscript:** Michel F.G. de Maat, Cornelis J.H. van de Velde, Martijn P.J. van der Werff, Hein Putter, Naoyuki Umetani, Elma Meershoek Klein-Kranenburg, Roderick R. Turner, J. Han J.M. van Krieken, Rob A.E.M. Tollenaar, Dave S.B. Hoon

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**Appendix**

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).