

Identification of a Quantitative *MINT* Locus Methylation Profile Predicting Local Regional Recurrence of Rectal Cancer

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Abstract

Purpose: Risk assessment for locoregional disease recurrence would be highly valuable in preoperative treatment planning for patients undergoing primary rectal tumor resection. Epigenetic aberrations such as DNA methylation have been shown to be significant prognostic biomarkers of disease outcome. In this study, we evaluated the significance of a quantitative epigenetic multimarker panel analysis of primary tumors to predict local recurrence in rectal cancer patients from a retrospective multicenter clinical trial.

Experimental Design: Primary tumors were studied from patients enrolled in the trial who underwent total mesorectal excision for rectal cancer ($n = 325$). Methylation levels of seven methylated-in-tumor (*MINT*) loci were assessed by absolute quantitative assessment of methylated alleles. Unsupervised random forest clustering of quantitative *MINT* methylation data was used to show subclassification into groups with matching methylation profiles.

Results: Variable importance parameters [Gini-Index (GI)] of the clustering algorithm indicated *MINT3* and *MINT17* (GI, 20.2 and 20.7, respectively) to be informative for patient grouping compared with the other *MINT* loci (highest GI, 12.2). When using this two-biomarker panel, four different patient clusters were identified. One cluster containing 73% (184 of 251) of the patients was at significantly increased risk of local recurrence (hazard ratio, 10.23; 95% confidence interval, 1.38-75.91) in multivariate analysis, corrected for standard prognostic factors of rectal cancer. This group showed a significantly higher local recurrence probability than patients receiving preoperative radiation ($P < 0.0001$).

Conclusion: Quantitative epigenetic subclassification of rectal cancers has clinical utility in distinguishing tumors with increased risk for local recurrence and may help tailor treatment regimens for locoregional control. *Clin Cancer Res*; 16(10); 2811-8. ©2010 AACR.

Rectal cancer is the second most common cancer of the digestive system in the United States (1). Worldwide, colorectal cancer has the second highest incidence (2), with rectal tumors constituting 33% of large bowel tumors (3). An important clinical feature of rectal cancer is its close anatomic relation to the small pelvis, which makes it prone to local recurrence after surgical removal even at early-stage disease. Locoregional recurrence occurs in

approximately 10% of rectal cancer patients after total mesorectal excision (TME) surgery with curative intent (4, 5). However, locoregional recurrence is difficult to treat, with a very poor overall survival prognosis.

Fixation in the small pelvis makes malignancies of the rectum suitable for external beam radiation therapy. The Dutch multicenter TME clinical trial showed significant reduction of local recurrence by adding short-course (5 × 5 Gy) preoperative radiation therapy to TME (6). The multidisciplinary treatment of rectal cancer is a subject of many clinical trials randomizing patients to regimens that include various neoadjuvant therapies combined with radical surgery (7-10).

(Neo)adjuvant regimens aim at two clinical outcome parameters: improvement of local control and/or reduction of distant recurrence, the latter occurring in approximately 25% of patients after radical primary tumor resection (4). The improved local control shown by the TME clinical trial has not translated into an overall survival benefit in the trial analyses after six years of follow-up, and the data show that overall survival is determined predominantly by distant recurrence (4). The trial data further

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Translational Relevance

The treatment of rectal cancer is multidisciplinary and combines radical surgery with preoperative radiotherapy, which has been shown to reduce local recurrence rates from 8% to 2.5%. However, radiotherapy comes with increased morbidity and reduced quality of life. This study suggests that quantitative measurement of methylation levels of methylated-in-tumor loci is predictive of local recurrence probability, and therefore may be helpful in preoperative selection of patients that will benefit from neoadjuvant radiotherapy.

show that only 25% of nonirradiated tumors with distant spread also recur locally, whereas 60% of the locally recurrent tumors show distant disease spread (4, 6). The distant-spreading, nonlocally recurring tumor may therefore constitute a separate subclass of rectal cancer.

Allocation of patients to neoadjuvant therapies might lead to overtreatment; because 10% of rectal cancer patients will develop local recurrence, 90% of patients may be overtreated. Neoadjuvant therapies used in the treatment of rectal cancer have their specific morbidities, as has been shown for both radiotherapy (11) and combined chemoradiotherapy (12). It is therefore of great importance to define biomarkers that can categorize tumors into local and distant-spreading type preoperatively to target the multimodality treatment regimens towards a more systemic or local approach.

Molecular analysis of primary tumor tissues is an attractive form of preoperative diagnostics because rectal primary tumors are easily accessible for biopsy, which is routinely done in the preoperative work-up. In contrast to colorectal cancer, to date few biomarkers have been described to be predictive or of prognostic value specifically in rectal cancer. It is important especially in multivariate analyses to distinguish rectal cancer from other bowel adenocarcinomas or colon cancer. Specific factors need to be taken into account for rectal cancer as compared with colon cancer, such as circumferential margin involvement, type of surgical procedure, or distance of the tumor to the anal verge (13).

Epigenetic changes, such as changes in DNA methylation status, are regarded as early events contributing to carcinogenesis (14). Methylation of cytosine residues in DNA is one of the mechanisms regulating transcriptional activity (15). In cancer, aberrant DNA hypermethylation of specific regions as well as global hypomethylation is observed (16). In this study, we investigated epigenetic changes in rectal cancer and specifically methylation of methylated-in-tumor (*MINT*) loci. *MINT* loci are CpG dinucleotide-rich regions located in nonprotein-encoding DNA regions and have been reported to become methylated in a tumor-specific manner in (colo)rectal cancer (17, 18), gastric cancer (19), and recently malignant melanoma (20).

In a previous study, we quantitatively studied methylation of *MINT* loci in premalignant stages of rectal cancer and showed that the *MINT* methylation index increases during adenomatous transformation of normal epithelium (18). We also identified a quantitative, two-*MINT* methylation biomarker panel (*MINT3* and *MINT17*) that is predictive of distant recurrence in early, node-negative rectal cancers patients (18). In the present study, we assessed the value of this panel to predict local recurrence of patients enrolled in the multicenter, randomized, clinical TME trial.

Materials and Methods

Tissue specimens. Primary paraffin-embedded archival tissue (PEAT) specimens were obtained from 325 nonirradiated patients enrolled in the TME multicenter clinical trial (4). Patients used in this study fulfilled the following criteria: nonirradiated, tumor-node-metastasis (TNM) stage I-III, with no evidence of disease after surgical resection. Using power calculations, a sample size of 250 patients was calculated to be sufficient to obtain statistical significance for predicting recurrence (with $\alpha = 0.05$ and a power of 90%), as described previously (18). Allowing for 30% loss of patient samples due to availability and quality of PEAT and DNA, 75 additional PEAT specimens were collected. In 11 patient PEAT blocks, tumor tissue was no longer present on the section. DNA was isolated from 314 randomly selected patient specimens with sufficient tumor cell numbers. After processing and sodium bisulfite treatment, samples of 251 patients yielded sufficient amount of quality DNA for absolute quantitative assessment of methylated alleles (AQAMA). The selected group of patients analyzed in our previous study (18) did not differ from nonselected patients in the nonirradiated treatment arm or from the complete trial population. Trial eligibility criteria and follow-up protocols have been described previously (4, 21, 22). Nonirradiated patients were selected because the predictive value of the tested biomarkers for local recurrence probability should be tested in patients who did not receive adjuvant therapy, as this affects local recurrence.

For external validation studies, 43 additional TME trial patients that adhered to the same selection criteria were added. Further, 42 nonirradiated patients who participated in the transanal endoscopic microsurgery (TEM; ref. 23) were added and these have been previously described (24). DNA was extracted from adenomatous and cancer tissues. The adenomas were subdivided into cases consisting of only adenoma tissue in the resection (A; $n = 21$) and adenoma fractions of cases with a carcinoma focus infiltrating at least in the submucosa (A, C+; $n = 8$). The carcinomas were subdivided into groups: tumor fractions consisting of a mixture of adenoma and carcinoma tissue (C+A; $n = 6$) and only carcinomas (C; $n = 7$). We further collected normal rectal epithelial tissue from the tumor specimen resection margins as normal controls from 19

patients operated for rectal cancer at the Saint John's Health Center.

DNA preparation from PEAT specimens. From the TME trial patient's primary tumor PEAT specimens, 7- μ m tissue sections were cut and mounted on nonadhesive glass slides. Tumor-representative areas on H&E-stained sections were identified and marked by a surgical pathologist specializing in rectal cancer (JHJMvK). Per patient, two tissues sections were deparaffinized. DNA was isolated from microdissected tissue from the marked areas and modified by sodium bisulfite, as previously described (25). Salmon sperm DNA was added as a carrier (26). Before and after sodium bisulfite modification, double-stranded and single-stranded DNA were quantified using PicoGreen and OliGreen assays (Molecular Probes; Invitrogen), respectively. Sufficient input DNA for AQAMA was determined as described (17). To assess background signal, a salmon sperm DNA sample without tumor DNA was included in all assays in triplicate. To prevent any bias, PEAT blocks and isolated DNA samples were coded.

AQAMA MINT locus methylation level assessment. Primary tumor methylation levels of *MINT* loci 1, 2, 3, 12, 17, 25, and 31 were assessed by AQAMA in triplicate (17). Controls for specificity of AQAMA for both methylated and unmethylated sequences, as well as controls for nonspecific amplification, were included (17, 27). As a final outcome of the analysis, the methylation index (MI) of a sample was the mean value of three MI measurements that were calculated

for each well as follows: $[\text{copy number}_{\text{methylated alleles}} / (\text{copy number}_{\text{methylated alleles}} + \text{copy number}_{\text{unmethylated alleles}})]$. Of these three measurements the SD was calculated. Values with a SD <0.1 were accepted and used in analysis. When the SD was >0.1 this was in most cases due to one or two of the three methylated or unmethylated measurements being undetermined. These cases were identified and the MI was then calculated as mean methylated copy number / (mean methylated copy number + mean unmethylated copy number). If one of three methylated or unmethylated measurements could not be determined this value was not incorporated in the calculation. If two or more of three measurements could not be determined the value was zero. Cases with six complete results and with SD > 0.1 were individually evaluated. When after omitting the most deviate MI value the SD was still >0.1 the sample was excluded from analysis.

Statistical analysis. Differences in recurrence probability, survival, and clinical and tumor pathologic factors were analyzed between TME trial patients assigned by unsupervised random forest (RF) clustering using *MINT3* and *MINT17* as described (18). Unsupervised RF clustering dissimilarity algorithms are based on individual decision tree predictors, and it automatically dichotomizes the expressions into clusters in a data-driven approach (28). Groups are therefore not established by employing cutoffs. The internal validation of RF clustering further eliminates the need for separate training and validation sets to test

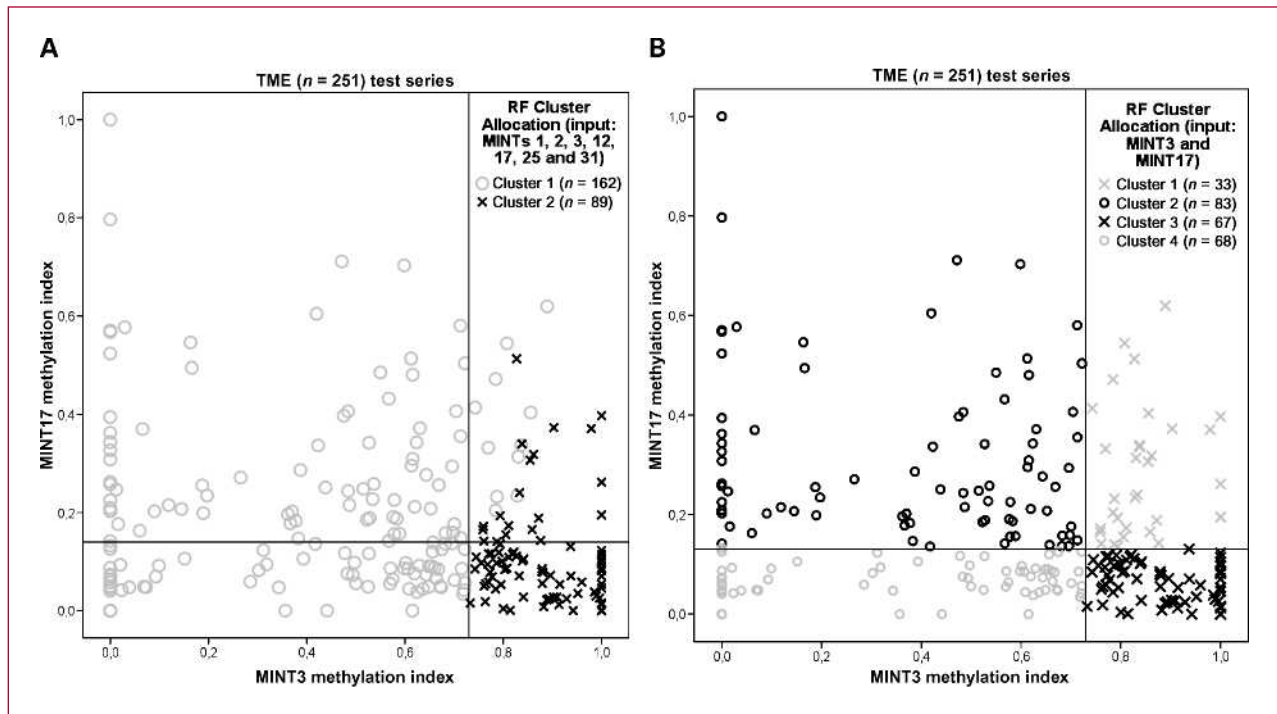


Fig. 1. Methylation index plots of *MINT3* and *MINT17* for individual patients. Representation of *MINT3* (X-axis) and *MINT17* (Y-axis) methylation indices for TME trial patients using all seven (A) and using only *MINT3* and *MINT17* (B) in the random forest algorithm, respectively. The horizontal and vertical reference lines represent cutoffs to separate the groups.

reproducibility of cluster formation. External validation of the data in an independent patient group is still required. The Gini-Index (GI; refs. 29, 30) of each input variable (*MINT* locus) was given. This index measures the inequality of two distributions and is defined as the ratio between the area spanned by the observed cumulative distribution and the area of a uniform hypothetical cumulative distribution for a nondiscriminating variable. A higher GI shows increasing inequality and therefore higher discrimination of that input variable.

Specimens yielding insufficient or low-quality DNA for PCR were excluded. To compare ordinal variables, Mann-Whitney and Kruskal-Wallis *U* tests were carried out. Differences in age were assessed using *t* tests. Cumulative incidences, accounting for death as a competing risk, were used to visualize survival differences (31), and significance was assessed by the log-rank test. For multivariate analysis the Cox proportional hazards model was used, with results presented as hazard ratios and 95% confidence intervals. Covariables entered in the model included T stage, N stage, circumferential margin status, distance of the tumor from the anal verge, and tumor differentiation. All clinical correlative analyses with identified clusters were done using SPSS statistical software (version 16.0.1, SPSS Inc.). A two-sided *P* value of ≤ 0.05 was considered statistically significant. Data on patients alive or free of recurrence were censored at the time of the last follow-up.

Results

***MINT* locus methylation profiling.** In our previous study, methylation levels at the seven *MINT* loci were measured in normal, adenomatous, and cancer rectal tissue (18). The results showed that in normal tissue, all *MINT* loci,

except for *MINT17*, were mostly unmethylated. Significantly higher methylation of *MINT2*, *MINT3*, and *MINT31* was detected in adenoma and cancer tissue compared with normal tissue. Further analysis of the quantitative data showed nonparametric distributions indicating the presence of subgroups for *MINT1*, *MINT2*, *MINT12*, *MINT17*, *MINT25*, and *MINT31* in adenoma and cancer tissue. Based on these findings, we concluded that all seven *MINT* loci had potential to subclassify rectal cancer patient groups with corresponding methylation level patterns. Quantitative methylation data of the seven *MINT* loci were then used to do unsupervised RF clustering analysis. An important aspect of RF clustering is that it eliminates a validation-training approach because of its internal validation quality. External validation of the data is still required. The multidimensional scaling plot as an outcome of using all seven *MINT* loci in the RF clustering algorithm suggested the presence of two clusters (18). The GI of the RF analysis, indicating variable importance, appointed *MINT3* and *MINT17* as the two *MINT* loci that carried the most information to form the clusters (18). GI being a measure of inequality, the higher the GI, the more the clusters can be considered different based on that specific biomarker. *MINT3* and *MINT17* were shown to have the highest GI (20.2 and 20.7, respectively), compared with the five other *MINT* loci (range, 6.0-13.5; ref. 18). The multidimensional scaling plot of the RF clustering using only these two biomarkers showed four clearly separate groups (18). Methylation level differences at the seven studied *MINT* loci between the four clusters are given in Supplementary Table S1. For a more simplified representation, individual patient MI values of *MINT3* and were rendered in XY-plots. In Fig. 1A the patients are labeled according to the outcome of the RF analysis using

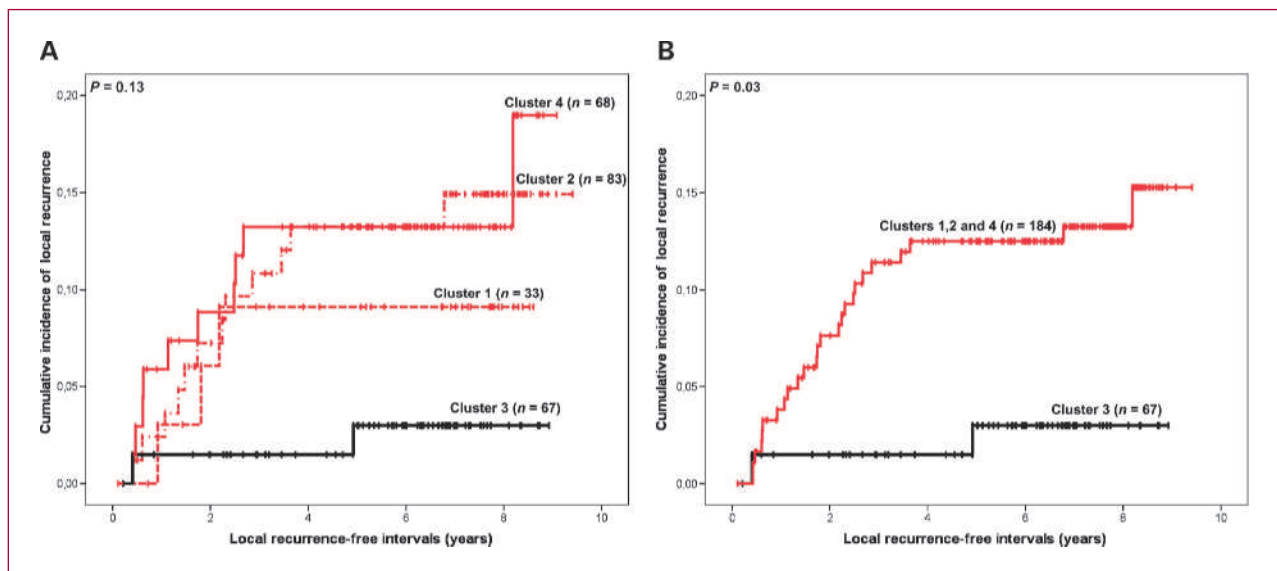


Fig. 2. Cumulative incidence plots showing differences in local recurrence rates among the four separate clusters (A) and distant for cluster 3 patients compared with patients in the combined clusters 1, 2 and 4 (B).

Table 1. Comparison of clinical and tumor pathology factors between identified two *MINT* locus clusters in all patients

	<i>MINT3</i> and <i>MINT17</i>		<i>P</i>
	Clusters 1, 2, and 4	Cluster 3	
	<i>n</i> = 184	<i>n</i> = 67	
Sex, <i>n</i> (%)			
Male	116 (72)	46 (28)	0.41
Female	68 (76)	21 (24)	
Age (y)			
Mean (SE)	62.7 (0.9)	65.0 (1.4)	0.17
TNM stage, <i>n</i> (%)			
I	51 (73)	19 (27)	0.78
II	57 (76)	18 (24)	
III	76 (72)	30 (28)	
N status, <i>n</i> (%)			
N ₀ (≥12 examined)	27 (79)	7 (21)	0.67
N ₀ /N _x (<12 examined)	82 (73)	30 (27)	
N ₁ (1-3 positive)	44 (72)	17 (28)	
N ₂ (≥4 positive)	32 (73)	12 (27)	
Differentiation, <i>n</i> (%)			
Well	14 (78)	4 (22)	0.90
Moderately	128 (73)	48 (27)	
Poor	42 (74)	15 (26)	
Location distant recurrences, <i>n</i> (%)			
Liver	19 (70)	8 (30)	0.44
Nonliver	24 (59)	17 (41)	
Resection type, <i>n</i> (%)			
Low anterior	125 (75)	42 (25)	0.46
Abdominoperineal	53 (69)	24 (31)	
Hartmann	6 (86)	1 (14)	
Circumferential margin, <i>n</i> (%)			
Negative	152 (73)	55 (27)	1
Positive	32 (73)	12 (27)	

seven *MINT* loci that showed the presence of two clusters. In Fig. 1B the patients are labeled according to the outcome of the RF analysis using only *MINT3* and *MINT17* that showed the presence of four clusters. Figure 1B further shows that the groups can be divided by a cutoff of MI = 0.73 for *MINT3* and MI = 0.14 for *MINT17* with almost no misclassification. This is important because such a two-dimensional cutoff algorithm can be used for validation experiments (see below). The two-biomarker cluster allocation of Fig. 1B continued to be used for the clinical correlation studies.

Univariate analysis of clinicopathologic parameters. Next, we were interested in comparing the probability of locoregional recurrence among the four clusters. As shown in Fig. 2A, the incidence of locoregional recurrence was lowest for patients in cluster 3. The quantitative methylation pattern of cluster 3 patients was that the tumors showed significantly higher methylation at *MINT3* and

lower methylation at *MINT17*. This pattern corresponds with the clinically relevant rectal cancer patient cluster at high risk for distant recurrence and decreased cancer-specific and overall survival that we previously identified (18). Clusters 1, 2, and 4 showed similar probability outcomes. The difference in local recurrence probability became more evident and reached statistical significance after combining clusters 1, 2, and 4 ($P = 0.03$; Fig. 2B). This result shows that the specific combination of increased methylation at *MINT3* and decreased methylation at *MINT17* is predictive of reduced local recurrence probability. From these data it was shown that quantitative methylation assessment of *MINT3* and *MINT17* identifies a patient group with an inversed risk incidence for distant recurrence and local recurrence.

Supplementary Table S1 shows the significance of MI differences between cluster 3 and the combined clusters 1, 2, and 4. It is shown that the 95% confidence intervals of *MINT3* and *MINT17* do not overlap, indicating that the rectal cancer patient groups' epigenetic classification is very distinct. The results also show that *MINT1* and *MINT2* can be helpful as discerning biomarkers for both patient groups.

Using univariate analysis, standard clinicopathologic parameters including sex, age, TNM stage, N status, tumor differentiation, location of distant recurrences, resection type, and circumferential margin status were compared between cluster 3 and the combined group of clusters 1, 2, and 4. These parameters did not significantly differ between the two patient groups identified based on methylation levels of *MINT3* and *MINT17* (Table 1). In addition, no significant difference was observed for these standard clinicopathologic factors between the patient groups when nodal status was taken into account (Supplementary Table S2).

Multivariate analyses. To assess whether the observed prognostic value of the clusters was independent of standard prognostic variables, we carried out multivariate analyses. The Cox regression method was used to analyze

Table 2. Multivariate analyses in all analyzed patients

Variable	Local Recurrence	
	HR (95% CI)	<i>P</i>
T stage (III-IV)	1.16 (0.44-3.08)	0.76
Node (+)	3.40 (1.39-8.33)	0.007
Circumferential margin (+)	2.27 (0.93-5.53)	0.07
Distance from anal verge <5 cm	1.40 (0.62-3.18)	0.42
Poor differentiation	0.98 (0.40-2.41)	0.96
<i>MINT</i> locus profile*	10.23 (1.38-75.91)	0.02

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

*"Cluster 3" is null-hypothesis.

standard prognostic factors of rectal cancer: T stage, N stage, circumferential margin status, distance of the tumor from the anal verge, and tumor differentiation (Table 2). Based on the epigenetic subclassification, the multivariate analysis showed patients of clusters 1, 2, or 4 to be at significant, >10-fold, increased risk of local recurrence compared with cluster 3 patients. Nodal involvement was further significantly associated with increased local recurrence incidence, and tumor involvement of the circumferential resection margin showed borderline significance.

Comparison with preoperatively irradiated tumors. Because we found the nonirradiated cluster 3 patients to be at reduced risk for local recurrence, we were interested to see how the recurrence probability rates of this group compared with those of preoperatively irradiated patients from the TME trial (Fig. 3). The irradiated patients were selected using the same clinical parameters that did not differ significantly from the nonirradiated selected patients (data not shown). The difference in the locoregional recurrence rate in cluster 3 patients was 3% versus 4.7% in patients who received 5×5 Gy before surgery ($P = 0.43$). Patients of high-risk cluster 1, 2, or 4 had significantly higher recurrence probability over time postoperatively compared with irradiated patients ($P < 0.0001$). These results indicate that patients with TME alone identified by the quantitative two-marker *MINT* profile have at least a comparable, locoregional recurrence rate compared with irradiated patients. This group will likely not be disadvantaged when preoperative radiotherapy before TME is not given and can be spared from unnecessary treatment morbidity.

External validation experiments. To test the reproducibility of cluster allocation by the cutoffs established in Fig. 1B we measured *MINT3* and *MINT17* methylation levels in primary tumor tissues of 43 additional TME trial patients,

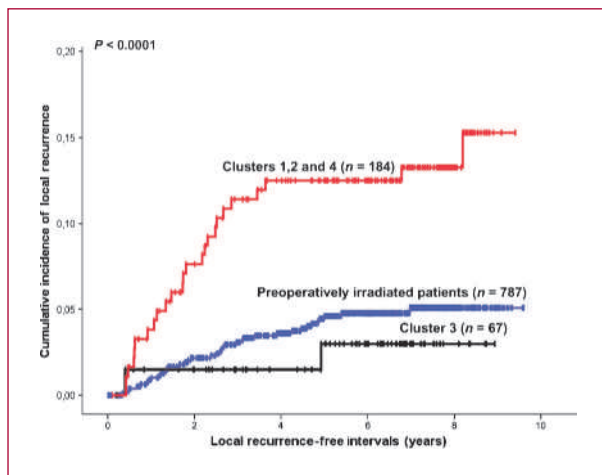


Fig. 3. Cumulative incidence plots displaying local recurrence incidence differences between irradiated and nonirradiated patients. Significant local recurrence incidence differences are seen between patients that received preoperative radiation and patients belonging to the identified high-risk cluster for local recurrence.

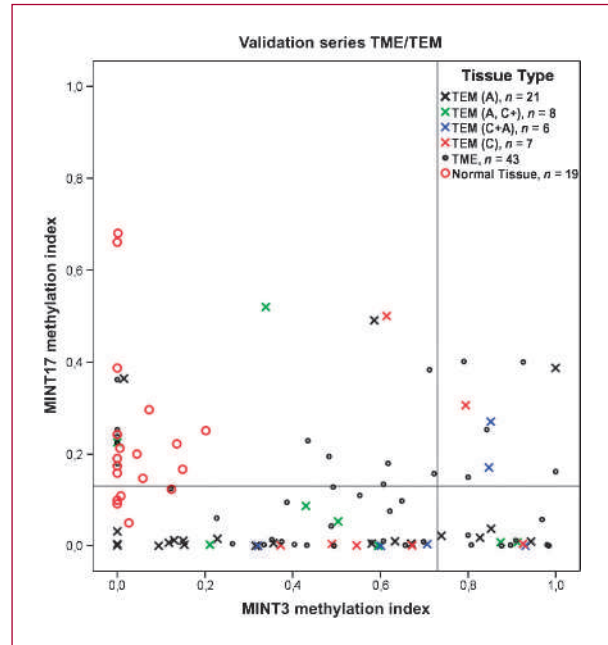


Fig. 4. Two-dimensional cutoff validation experiment results.

Representation of *MINT3* (X-axis) and *MINT17* (Y-axis) methylation indices for patients of the TME trial, TEM study, and 19 normal rectal mucosa samples. The horizontal and vertical reference lines represent the previously suggested cutoffs to separate the groups. For the TEM series, the following specimens were assessed: adenoma with no carcinoma cells (A); adenoma with carcinoma cells (A, C+); carcinoma mixed with adenoma cells (C+A); and carcinoma (C).

and an independent group of 42 patients consisting of rectal adenomatous and cancer tissue from patients treated by TEM. Figure 4 shows the results, with 19% (8 of 43) of TME and 19% of TEM patients allocated to the prognostic cluster 3. The suggested cutoffs therefore show allocation of an independent group of rectal cancer patients, including patients with adenomas and very early disease stage, consistent in size and comparable with the size of the test group patients (27%). In the additional TME patient group there was only one event for local recurrence (which did occur in the high-risk cluster). The TEM group was treated differently and also contained premalignant stages and therefore was not comparable. Further external validation of these clinical findings in future studies is needed.

To show the MI of the two biomarkers in normal rectal mucosa we added those data of 19 specimens to Fig. 1B. The results of the normal rectal tissue analysis shows that these are well separable from a cluster 3 patient, which is important as these patients may be selected for treatment of their rectal tumor by surgery alone.

Discussion

Although recurrence rates have decreased to about 10% after the introduction of TME surgery, locally recurrent

cancer remains an important clinical problem (1). In a previous study (18), *MINT* methylation was shown to increase early during tumor progression, indicating that methylation of *MINT* loci is a factor acquired early during rectal tumorigenesis, and therefore can be used to subclassify early disease. Preoperative molecular profiling of the primary tumor could potentially be of great value in identifying patients at high risk of developing local recurrence. This study shows that based on absolute quantitative methylation levels of the *MINT3* and *MINT17* loci, rectal cancers with a high risk of local recurrence can be identified.

The *MINT3* locus CpG island is localized on chromosome 1p34-35 just downstream of *RBBP4* (retinoblastoma-binding protein 4; ref. 32), *SYNC1* (encoding the syncoilin protein involved in cell cytoskeleton and extracellular matrix proteins; ref. 33), *YARS* (tyrosyl-tRNA synthetase, involved in angiogenesis; refs. 34, 35), and *s100p*-binding protein (*s100p* is overexpressed in many solid tumors; refs. 36, 37). *MINT17* is localized on the long arm of chromosome 12, just upstream of the *Harakiri* (*HRK*) gene, a member of the *BCL2* gene family, which encodes apoptosis regulatory proteins and its expression in gastrointestinal cancers is known to be regulated by promoter region methylation (38). A regulatory or functional relation between the *MINT3* and *MINT17* CpG islands and the above mentioned genes has not been established. *MINT* locus methylation may therefore be seen as a surrogate biomarker. In the literature, several studies have shown the clinical utility of *MINT* loci methylation as a predictive biomarker in colon and gastric cancer, melanoma, and renal cell carcinoma (18, 39–42). Our large-scale study adds novel findings that *MINT* locus methylation levels may have utility in predicting rectal cancer local recurrence. Importantly, in this study we propose an algorithm using only two *MINT* biomarkers with a simple cutoff. We showed validation of the algorithm to have excellent clustering capacity, even in early-stage disease, and the ability to separate cases well from normal rectal tissue.

This is the second study specific for rectal cancer from our group that shows the clinical utility of *MINT3* and *MINT17* methylation levels. Strong probability differences between the two patient groups could be shown for local recurrence, with cluster 3 patients having >10-fold increased risk of developing local recurrences than patients allocated to the other clusters. Local recurrence rates of cluster 3 patients were comparable with those of irradiated rectal cancer patients, and this shows that leaving out preoperative radiation can be done safely with the advantage of reducing treatment morbidity. This molecular stratification approach needs to be further investigated in a randomized multicenter trial to be validated.

Subdivision according to nodal status (data not shown) showed that patients in cluster 3 with a positive nodal status were at significantly increased risk of local recurrence. Node-negative patients in cluster 3, however, showed a significantly decreased cancer-specific and overall survival compared with the other clusters in our previous study

(18). This is explained by the fact that these node-negative patients were found to be at significantly increased risk of distant recurrence in our previous study (18). Early metastasizing of rectal cancer, in the absence of evident nodal spread, may occur via the hematogenic route. This is supported by our group that circulating tumor cells can be detected in peripheral blood of early stage I/II colorectal cancer patients which has prognostic clinical utility (43). Our findings are in accordance with data from the TME trial showing that survival is determined predominantly by distant and not by locoregional recurrence (4). This suggests that nonlocally recurrent and distantly spreading rectal cancer constitutes a separate subclass of rectal cancers that can be identified by our two-biomarker *MINT* methylation profile.

The TME trial showed reduction of rectal cancer local recurrence rates by adding preoperative radiotherapy (7). A reduction of distant recurrence of rectal cancer, as well as improved disease-free and overall survival, has been shown in several randomized controlled trials, either alone or in combination with radiotherapy (44–46) using adjuvant chemotherapy. This is the first study to show that locoregional recurrence patterns of American Joint Committee on Cancer stage I, II, and III rectal cancers can be distinguished using preoperatively assessable, quantitative epigenetic subclassification of primary rectal tumor tissue. Our previous study showed the methylation status of the described *MINT* loci to have utility in predicting distant recurrence probability in stage I and II disease. Therefore, a new treatment stratification approach for rectal cancer can be suggested as follows: after preoperative assessment of primary tumor biopsy specimens of *MINT3* and *MINT17* methylation levels, about 30% of patients could be spared from preoperative radiation therapy, but might benefit from systemic treatment. The other 70% should receive preoperative radiotherapy, and if node-positive, postoperatively systemic treatment can be considered. The identification of patients who do not need preoperative radiotherapy would likely reduce long-term morbidity and improve quality of life (47). The results of our study should be further evaluated to improve planning of therapeutic regimens for rectal cancer patients in the preoperative phase.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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