Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/32781 holds various files of this Leiden University dissertation.

Author: Benard, Anne
Title: Epigenetic prognostic biomarkers in colorectal cancer
Issue Date: 2015-04-21

Chapter 2

Epigenetic status of LINE-1 predicts clinical outcome in early-stage rectal cancer

Anne Benard, Cornelis J H van de Velde,
Laurent Lessard, Hein Putter, Ling Takeshima,
Peter J K Kuppen, Dave S B Hoon

2

Abstract

Background: We evaluated the clinical prognostic value of methylation of two non-coding repeat sequences, long interspersed element 1 (LINE-1) and Alu, in rectal tumor tissues. In addition to DNA methylation, expression of histone modifications H3K27me3 and H3K9Ac was studied in this patient cohort.

Methods: LINE-1 and Alu promoter methylation was assessed in DNA extracted from formalin-fixed paraffin-embedded tissues. A pilot (30 tumor and 25 normal tissues) and validation study (189 tumor and 53 normal tissues) were performed. Histone modifications H3K27me3 and H3K9Ac were immunohistochemically stained on tissue microarrays of the study cohort.

Results: In early-stage rectal cancer (TNM stage I-II), hypomethylation of LINE-1 was an independent clinical prognostic factor, showing shorter patient survival (p=0.014; HR 4.6) and a higher chance of tumor recurrence (p=0.001; HR 9.6). Alu methylation did not show any significant correlation with clinical parameters, suggesting an active role of LINE-1 in tumor development. Expression of H3K27me3 (silencing gene expression) and H3K9Ac (activating gene expression) in relation to methylation status of LINE-1 and Alu supported this specific role of LINE-1 methylation.

Conclusion: The epigenetic status of LINE-1, but not of Alu, is prognostic in rectal cancer, indicating an active role for LINE-1 in determining clinical outcome.

Introduction

Under the current TNM (tumor, node, metastasis) staging system (American Joint Committee on Cancer, AJCC (1)) and treatment guidelines (National Cancer Institute, NCI), there is both over- and undertreatment of rectal cancer patients (2). In our search for biomarkers that can complement the current TNM staging system as well as aid in subsequent treatment decisions for rectal cancer patients, we assessed genome-wide DNA methylation using repeat sequences LINE-1 (long interspersed element) and Alu, and global histone modifications in rectal tumor tissues.

Changes in epigenetic mechanisms regulating gene expression, including DNA methylation of CpG dinucleotides, are major factors in tumorigenesis (3). We have previously reported that epigenomic aberrations play a significant role in tumor progression and clinical outcome in rectal cancer (4-6). These studies showed that methylation of non-coding regions can be used as prognostic biomarkers in colorectal cancer. Human DNA contains large numbers of non-coding repeat sequences, the most studied sequences being LINE and Alu repeats. LINE-1 repeat sequences constitute about 17% of the total human genome, are present on most of the chromosomes, and comprise about 50 different subfamilies (7). Alu repeats constitute about 11% of the human genome (8). Because of their abundance in the human genome, methylation of LINE-1 and Alu sequences has been used as surrogate for genome-wide DNA methylation status (9). Hypomethylation of both LINE-1 and Alu sequences has been associated with malignancies (10), including sporadic cases of hemophilia (11) and several types of solid tumors (12-18). In addition to DNA methylation, histone modifications play critical roles in regulating gene expression. Gene expression is dependent on the presence of transcription factors, and mostly on the access of these transcription factors to the transcription start sites. The chromatin structure surrounding the transcription start site, determined by the distance between individual nucleosomes comprised of DNA wrapped around histone octamers, is determined by both DNA methylation and histone modifications and determines accessibility of transcription factors to the DNA. DNA methylated at CpG sites in combination with "silencing" histone modifications, including trimethylation of lysine 27 on histone H3 (H3K27me3), is associated with a closed chromatin structure with limited access for transcription factors to the DNA (illustrated in Rodenhiser *et al.*; 19). Unmethylated DNA in combination with "active" histone modifications, including acetylation of lysine 9 on histone H3 (H3K9Ac) is associated with an open chromatin structure, which permits for gene transcription (19).

Given the recognized de-regulation of these epigenetic mechanisms in cancer (3,20), we investigated whether both global DNA methylation and histone modification patterns can be used to predict clinical outcome in rectal cancer patients enrolled in a well-defined, strictly quality-controlled clinical trial (21).

Materials and methods

Patient selection

Formalin-fixed paraffin-embedded (FFPE) specimens were collected from patients enrolled in the Dutch TME multicenter clinical trial (non-irradiated arm) between 1996 and 1999 with

no evidence of disease after surgical resection and a median follow-up of 7 years. Patient characteristics are displayed in Table 1. Informed consent was obtained from all patients enrolled in the TME trial. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. Western IRB permission was obtained for assessment of patient specimens at John Wayne Cancer Institute. A pilot study was performed using 30 primary rectal tumor FFPE specimens, TNM tumor stages I (n=10), II (n=12) and III (n=8), and 25 normal rectal tissues resected at least 5 cm away from the tumor. TNM tumor stages were defined according to the American Joint Committee on Cancer, AJCC staging system (1).

For the validation study, a patient selection (n=189) representative for the non-irradiated arm of the TME trial was made based on sex, age at randomization, and circumferential margin involvement, with TNM tumor stages I (n=53), II (n=43), III (n=85) and IV (n=8). 53 additional normal rectal tissues were collected, with 45 out of the 53 cases having matching primary tumor specimens included in the study. Histone modifications were assessed on 132 tumor tissues and 50 normal tissues included in the validation cohort of the LINE-1 study and present on a tissue microarray (described below). This study was performed according to the REMARK guidelines (NCI-EORTC, (22)).

DNA extraction from FFPE specimens and quantification of DNA for methylation assays

Tumor areas on H&E-stained sections of FFPE tumor specimens were identified and marked by a pathologist. From each patient block, two 7μm FFPE sections were deparaffinized and stained with hematoxylin, followed by needle microdissection of tumor areas. DNA was extracted using a proteinase K-based protocol as described previously (23) and quantified using a Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR, USA).

Bisulfite conversion and quantitative real-time PCR for methylation assays

For each sample, 200ng of DNA was bisulfite-converted using the Epitect Bisulfite Kit (Qiagen; Valencia, CA); converted DNA was eluted in 50μl elution buffer. Both LINE-1 and Alu PCR-based assays have been described previously (24,25). Quantitative real time PCR for LINE-1 sequences (according to the MIQE guidelines; 26) was performed using universal primers and a locked nucleic acid (LNA) probe specific to the bisulfite-converted Methylated or Unmethylated LINE-1 consensus L1.2 sequence. Primer and probe sequences were as follows: LINE-1 Forward 5'-GGGTTTATTTTATTAGGGAGTGTTAGA-3', LINE-1 Reverse 5'-TCACCCCTTTCTT TAACTCAAA-3', LINE-1 FAM-M-Probe (LNA): 5'-TG+CG+CGAGT+CGAAG-3', LINE-1 FAM-U-Probe (LNA): 5'-TG+TG+TGAG+T+T+GAA+GTAG-3'. Thermal cycling reactions were as follows: hot start for 3 minutes at 95°C, followed by 45 cycles of denaturing at 95°C (15 seconds) and annealing/extension at 60°C (1 minute). For Alu repeat sequences, quantitative real time PCR was performed as described previously (27). Primer and probe sequences specific for the Alu consensus sequence were as follows: Alu Forward: 5'-GTTTGGTTAATATGGTGAAATT-3', Alu Reverse: 5'-ATTCTCCTACCTCAACC-3', Alu FAM-M-Probe (LNA): 5'-A+AC+GCGCGCCAC-3', Alu FAM-U-Probe (LNA): 5'-AAC+AC+A+CACCA+CCA-3'. Thermal cycling reactions were as described for LINE-1, but with annealing/extension at 58°C. Quantitative PCR reactions were run on a 384 CFX

TABLE 1. Patient characteristics of the pilot and validation study groups.

		All patients non-irradiated arm TME trial (n=769)		Pilot study (n=30)		Validation study (n=189)	
		n	**(%)**	**n**	**(%)**	**n**	**(%)**
Sex							
	Male	488	63.5	16	53.3	129	68.3
	Female	281	36.5	14	46.7	60	31.7
Age at randomization							
	Mean	64.4		64.1		63.9	
	Standard error	0.405		2.30		0.811	
TNM stage							
	I	224	29.6	10	33.3	53	28
	II	195	25.8	12	40	43	22.8
	III	281	37.2	8	26.7	85	45
	IV	56	7.4			8	4.2
Circumferential margin							
	Negative	621	80.8	29	96.7	152	80.4
	Positive	148	19.2	1	3.3	37	19.6
Tumor location							
	Rectum	679	90.1	26	86.7	161	85.6
	Anal region	54	7.2	3	10	20	10.6
	Other	21	2.8	1	3.3	7	3.7

Patient characteristics are shown for both the pilot and validation study groups. Patient selection for the pilot study was based on TNM stage. Patient selection for the validation study was based on availability of FFPE tissues and all listed variables, i.e. sex, age at randomization, TNM stage, circumferential margin involvement and tumor location. The validation study selection was representative for the entire non-irradiated patient cohort.

thermal cycler (BioRad, Benicia, CA). A serial dilution of plasmids with either the methylated or the unmethylated sequence was used to generate standard curves for quantification. Triplicates of each sample (20ng DNA per reaction) were run on the same plate, and mean copy numbers (CN) were used for statistical analyses. Measurements were repeated when triplicates varied >2 Cq values. Controls used in both LINE-1 and Alu methylation assays were universally methylated DNA (UMC; Millipore, Billerica, MA), universally unmethylated DNA obtained by repeated whole genome amplification of PBL DNA (UUC; Repli-g kit Qiagen, Valencia, CA), and peripheral blood lymphocytes. Specific methylated cell line controls for LINE-1 methylation assays were melanoma cell line M12 and for Alu methylation assays breast cancer cell line MCF-7. Methylation percentages were calculated as follows: methylation percentage = CN methylated / (CN methylated + CN unmethylated) * 100%.

2

Immunohistochemical staining and semi-automated scoring of histone modifications

Tumor and normal tissue FFPE blocks with enough tissue available were selected from patients enrolled in the non-irradiated arm of the Dutch TME clinical trial. Tumor cores from 496 patients, and normal tissue cores (taken at least 5cm away from the tumor) from 334 patients enrolled in the Dutch TME clinical trial were included to construct a tissue microarray (TMA). From each donor block, three 1-mm2 tissue cores from strictly tumor areas or one tissue core from normal tissue areas (center) as marked by an experienced pathologist were punched and transferred into a recipient paraffin block using a TMA Master (3DHistech, Budapest, Hungary). 4.5μm tissue sections of the TMAs were cut and processed for immunohistochemical staining. TMA sections were stained using mouse anti-H3K27me3 (dilution 1:200; ab6002, Abcam, Cambridge, UK) or rabbit anti-H3K9Ac (dilution 1:600, ab8898, Abcam, Cambridge, UK), using a standard IHC protocol (28). Briefly, antigen retrieval was performed by heating the sections for 10 min at 95°C in a citrate buffer (pH 6.1; pH low Target Retrieval Solution, Dako, Heverlee, Belgium) after deparaffinization. Endogenous peroxidase was blocked by incubating the sections in a 0.3% solution of hydrogen peroxide (in PBS) for 20 min. Sections were incubated with primary antibodies overnight (16 hrs). Immunohistochemical staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Heverlee, Belgium). Immunohistochemically stained tissue microarrays were scanned at 20x magnification on the Ariol system (Leica Microsystems, Rijswijk, The Netherlands). Expression of histone modifications was scored using the semi-automated Ariol system. Tumor areas were marked on the computer upon visual inspection, followed by training of the system to correctly identify positively stained and negative nuclei in the tissues, for each of the markers separately. Intensity of the staining was measured by the Ariol system and divided into three categories. The Ariol system was trained to count the number of cells in each category and to calculate a mean intensity score for each tissue core. Patients were divided into high and low intensity groups for each histone modification based on the median intensity score.

Statistical analysis

Statistical analysis of the data was performed in consultation with a biostatistician (H.P.) using statistical software SPSS version 20.0 (PASW Statistics, IBM Statistics, New York). Differences in mean LINE-1 methylation between the different TNM tumor stages were calculated using a Student's t-test (data were normally distributed). Univariate Cox Regression analyses were performed to calculate differences in survival time and tumor recurrence between patients with a high or a low methylation index (LINE-1 and Alu) or staining intensity (histone modifications). Cutoff points for the division of patients into the different patient groups were based on median intensity for the histone modifications and on median methylation index for LINE-1 or Alu. For histone modification analyses, only patients included in the LINE-1 validation study were used (tumors n=132, normal tissues n=63). For each patient, three cores (either three tumor or three normal cores) were scored and mean intensity scores were calculated for each patient. Tumor cores with non-specific staining were excluded from the analyses (n=13). Kaplan-Meier survival curves were made to visualize survival differences between high and low methylation groups (LINE-1) and high and low staining intensity (histone modifications) and statistical significance was assessed using the log-rank test. All time-to-event variables were calculated

from the date of surgery. Competing risk analyses were performed for cancer-specific survival and recurrence analyses in order to prevent overestimation of the number of events in each of the categories. Multivariate analyses using LINE-1 methylation or staining intensity of the histone modifications as continuous data were performed using the Cox proportional hazards model. Covariates entered in the multivariate model were age at time of surgery, circumferential margin, TNM-stage and tumor location. For all analyses, a two-sided p-value of 0.05 or less was considered statistically significant. Data were censored when patients were alive or free of recurrence at their last follow-up date.

Results

## Methylation assay verification

To ensure a good performance and reproducibility of the LINE-1 and Alu methylation assays, we performed several quality checks. To verify the reproducibility of the assays, we ran a test set of 8 patient samples for both Alu and LINE-1 methylation assays, which were independently repeated. Variation in methylation percentages between the two experiments was 0.1-1.6% for LINE-1 and 0.1-1.2% for Alu. Control samples included on every plate showed minimal inter-plate variability (<7%). Standard deviations were 6.6% for UMC and 3.8% for PBL in the LINE-1 methylation assays and 4.3% for MCF-7 and 5.4% for UMC in the Alu methylation assays. The limit of detection of this assay was as low as 100 copies of the respective plasmids in each reaction. Standard curves using plasmids of either the methylated or unmethylated sequences were highly reproducible for all assays, with R^2 values of 0.947 or higher between the duplicates on each of the individual plates. Taken together, the LNA-probe-based real-time PCR method used in this study proved to be very robust and reproducible.

LINE-1 methylation

The pilot study for LINE-1 methylation showed decreasing levels of methylation with increasing TNM tumor stage (Figure 1A). Normal tissues showed the highest LINE-1 methylation percentage as compared to tumor tissues, with an average difference between matching tumor and normal samples (n=8) of 14.2%. Levels of LINE-1 methylation were significantly different across TNM tumor stages and between normal and tumor samples (normal vs T1, p=0.024; across TNM tumor stages, p<0.001).

In the validation study, decreasing levels of methylation were observed with increasing TNM tumor stage (p<0.001) (Figure 1B). The mean difference between the matching normal and tumor samples (45 out of 53) was 21.6%, with a consistently higher methylation percentage in the normal samples. Univariate analyses showed low levels of LINE-1 methylation to be correlated with shorter survival (p=0.006; HR=5.169) and higher chance of distant recurrence (p=0.003; HR=9.943) in stage I and II rectal cancer patients (Table 2). Stage III and IV patients did not show any significant correlation of LINE-1 methylation with survival and recurrence. Subsequent analyses were done with stage I and II patients only. Multivariate analyses of the validation study data showed LINE-1 methylation status to be an independent predictor of survival (p=0.014; HR 4.568) and distant recurrence (p=0.001; HR 9.576) in early-stage rectal

Figure 1

A LINE-1 methylation - Pilot study

B LINE-1 methylation - Validation study
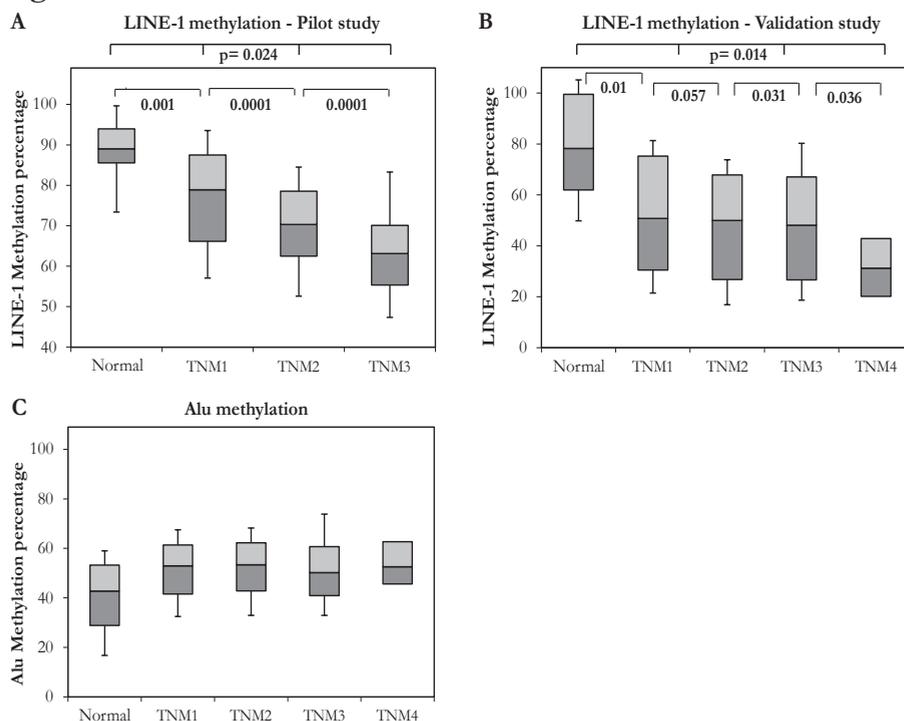
C Alu methylation

Mean LINE-1 methylation percentages according to TNM tumor stage in rectal cancer patients. *Mean methylation percentages are shown for the different TNM tumor stages. Methylation percentages were calculated as follows: methylated copy number / (methylated copies + unmethylated copies) * 100%. **A**. Mean LINE-1 methylation percentages are shown for different TNM tumor stages of the pilot study samples. The different TNM tumor stages are indicated as I, II and III, normal samples are indicated as 'normal'. **B**. and **C**. Mean LINE-1 and Alu methylation percentages, respectively, are shown for different TNM tumor stages of the validation study samples. The different TNM tumor stages are indicated as I, II, III and IV, normal samples are indicated as 'Normal'.*
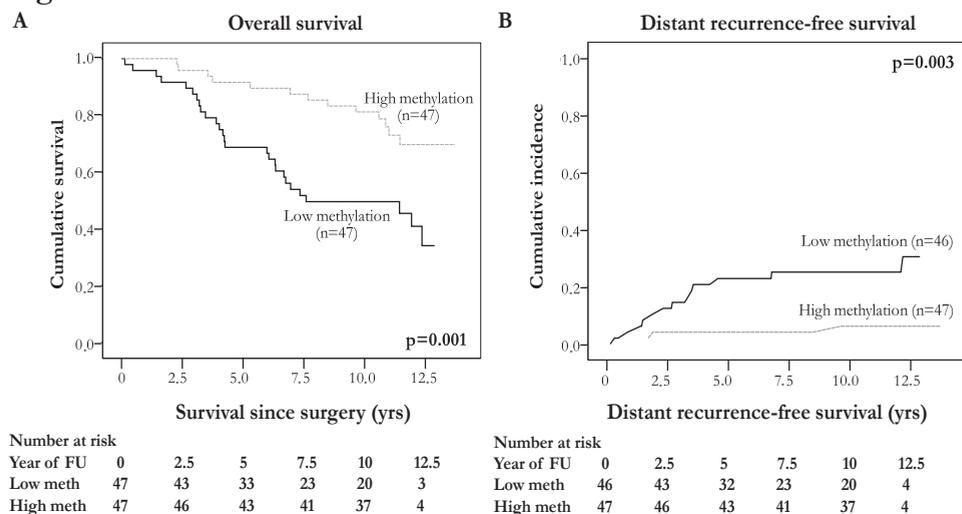
cancer patients (Table 2). Kaplan-Meier survival and cumulative incidence curves were generated (Figures 2A and 2B) to visualize differences in survival and recurrence between high and low LINE-1 methylation groups, based on median methylation percentage (57.4%) of stage I and II patients in the validation study. The log-rank test showed that patients with low LINE-1 methylation have shorter survival times and a higher chance of tumor recurrence than patients with a higher methylation percentage. Significant differences were observed for overall survival (p=0.006), overall recurrence (p=0.0017) and distant recurrence-free survival (p=0.003). No difference was found for local recurrence (p= 0.95), which can be explained by the fact that survival is mainly determined by distant metastases rather than local recurrences. The multivariate analyses showed that patients with a low level of LINE-1 methylation have a 9-fold higher chance of distant recurrence of the tumor and a 5-fold lower chance of survival than patients with a high LINE-1 methylation level (Table 2). No correlation was found between LINE-1 methylation and lymphocyte infiltration (p=0.22), a factor known to impact clinical outcome in colorectal cancer. Our data showed that LINE-1 methylation status was prognostic for disease outcome,

TABLE 2. Univariate and multivariate analyses for both patient survival and tumor recurrence in patients with stage I and II rectal tumors.

	Overall Survival		Local recurrence		Distant recurrence	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
LINE-1 univariate analysis						
LINE-1 methylation	5.239 (1.589-17.268)	**0.006**	0.892 (0.0.23-34.803)	0.951	9.943 (2.476-30.028)	**0.003**
LINE-1 multivariate analysis						
Age	1.028 (0.997-1.061)	*0.080*	0.931 (0.829-1.045)	0.223	0.957 (0.913-1.003)	*0.068*
Circumferential margin	1.352 (0.380-4.817)	0.642	0.513 (0.164-2.839)	0.962	1.778 (0.196-16.168)	0.609
TNM	1.570 (0.814-3.028)	0.178	0.230 (0.096-1.469)	0.964	1.907 (0.607-5.995)	0.269
Tumor location	1.336 (0.643-2.776)	0.437	0.346 (0.123-1.896)	0.974	4.606 (1.932-10.983)	**0.001**
LINE-1 methylation	4.568 (1.359-15.351)	**0.014**	1.438 (0.14-146.4)	0.877	9.576 (4.443-47.131)	**0.001**
Alu univariate analysis						
Alu methylation	1.269 (0.681-2.361)	0.453	1.093 (0.154-7.763)	0.929	1.897 (0.689-5.227)	0.215
Alu multivariate analysis						
Age	1.391 (1.016-1.904)	**0.039**	0.483 (0.156-1.494)	0.207	0.696 (0.451-1.072)	0.100
Circumferential margin	1.135 (0.316-4.077)	0.846	1.978 (0.699-5.604)	*0.080*	0.735 (0.083-6.500)	0.782
TNM	1.209 (0.697-2.097)	0.500	0.245 (0.034-1.780)	0.165	1.462 (0.573-3.731)	0.427
Tumor location	1.199 (0.579-2.484)	0.625	0.203 (0.029-1.543)	0.989	2.332 (1.046-5.201)	**0.038**
Alu methylation	0.996 (0.979-1.014)	0.690	0.985 (0.928-1.045)	0.613	0.980 (0.952-1.010)	0.185

*Survival analyses are shown for stage I and II patients only. The total number of patients included in the analyses was n=96. Hazard ratios are displayed for both Cox proportional hazard univariate and multivariate analyses and 95% confidence intervals are given for LINE-1 methylation. In multivariate analyses, age was entered as a continuous variable. For circumferential margin the 'negative' group was used as reference, for TNM the stage I group was used as reference. Tumor location was divided into the categories rectum, anal region, or other, using rectum as the reference category. Statistically ignificant values are indicated in **bold**, trends in Italic.*

Figure 2

A

B

Survival curves of stage I and II rectal cancer patients by LINE-1 methylation status. *Kaplan-Meier curves were made to visualize differences in survival and recurrence between patients with high and low LINE-1 methylation status. Log-rank tests were performed to calculate the difference between the low and high LINE-1 methylation groups. Survival times were calculated as time from surgery till an event (death or recurrence, respectively).* **A**. *Kaplan-Meier curves showing overall survival of the high and low methylation groups based on LINE-1 methylation status. Patients at risk are displayed for each of the groups.* **B**. *Cumulative incidence curves showing distant recurrence in the high and low methylation groups based on LINE-1 methylation status. Patients at risk are displayed for each of the groups.*

but Alu methylation was not. Chi-square (p=0.38) and Pearson's correlation (p= -0.306) analyses confirmed that Alu methylation and LINE-1 methylation indeed did not correlate. Similarly, in multivariate analyses, the interaction between Alu and LINE-1 methylation did not show significant correlation with survival (p=0.23). This confirmed that Alu and LINE-1 methylation did not have a similar effect on patient survival and tumor recurrence in this set of rectal cancer patients, suggesting that LINE-1 may be involved in specific tumor progression events that affect disease outcome in rectal cancer, rather than reflecting a genome-wide methylation status.

Alu methylation

To investigate if LINE-1 methylation did indeed represent genome-wide methylation status, we studied methylation of Alu repeat sequences in the same patient cohort. The levels of Alu methylation were not significantly different between normal and tumor tissues (pilot study; p=0.24) or between patients with or without a recurrence (pilot study; p=0.27). Alu methylation levels did not differ between the different TNM tumor stages in either the pilot (p=0.11) or the validation study (p=0.73; Figure 1C). The mean difference in Alu methylation percentage between normal and matching tumor tissues was 1.03% in the validation series.

Survival analyses on the data in the validation series did not show any significant differences using a cutoff based on median Alu methylation percentage (60%). Neither overall survival nor overall recurrence did show differences between the groups (p=0.65 and p=0.31, respectively).

Selection of stage I and II patients only, as described for the LINE-1 methylation studies, did not change outcomes of either univariate or multivariate survival analyses (Table 2).

Histone modifications

Histone modifications H3K27me3 and H3K9Ac were assessed by IHC on TMA sections. Only patients included in the LINE-1 validation study were used for histone modification analyses (tumors n=132, normal tissues n=63). Semi-automated analyses using the Ariol System yielded intensity scores for each of the individual tumor cores. Correct identification of positive and negative cells by the Ariol system is shown in Figure 3. Mean intensity scores of three tumor tissue cores were calculated for each patient and used for survival analyses. Patients were divided into high and low intensity groups for each histone modification based on the median intensity score. H3K27me3 intensity scores ranged from 0 - 63.14, H3K9Ac intensity scores ranged from 0 - 67.84. Median intensity scores were 50.59 in normal tissues and 48.20 in tumor tissues for H3K9Ac (p=0.053) and 49.41 in normal tissues and 51.77 in tumor tissues for H3K27me3 (p=0.002). H3K9Ac and H3K27me3 intensities in tumor tissues showed an inverse correlation, with p=0.024.

Figure 3

H3K9Ac
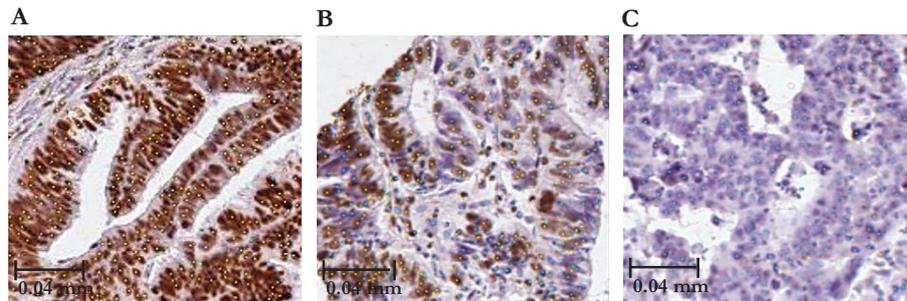
H3K27me3
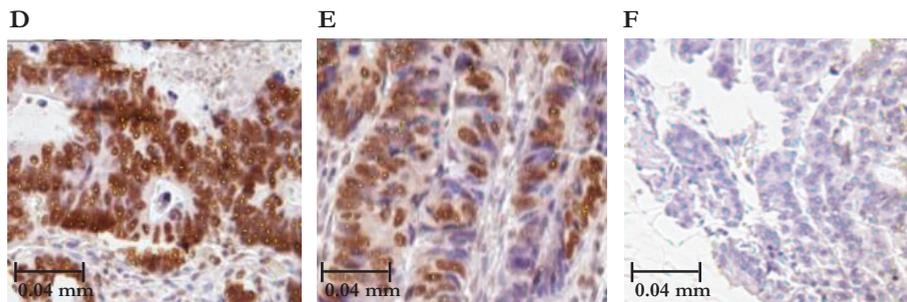
Identification of positively stained and negative tumor cells by the Ariol system. *The Ariol system trainer overlay shows correct identification of positive (yellow dots) and negative (blue dots) cells on tumor cores. TMA slides were scanned using a 20x magnification. Shown for both H3K9Ac and H3K27me3 are highly positive tumor cores (**A** and **D**), tumor cores with both positive and negative cells (**B** and **E**) and negative tumor cores (**C** and **F**). The Ariol system was trained to identify positive and negative cells for each individual marker.*

TABLE 3. Univariate and multivariate analyses for histone modifications in rectal cancer patients.

	H3K27me3		H3K27me3		H3K9Ac	
	LINE-1 methylation low		*LINE-1 methylation low*		*LINE-1 methylation high*	
	Overall Survival		Local recurrence-free survival		Local recurrence-free survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analysis						
Intensity histone modification staining	0.461 (0.236-0.901)	**0.024**	0.118 (0.014-0.960)	**0.046**	1.066 (0.974-1.261)	0.163
Multivariate analysis						
Age	1.070 (1.030-1.113)	**0.001**	1.044 (0.948-1.134)	0.274	1.012 (0.937-1.088)	0.739
Circumferential margin	2.003 (0.696-5.768)	0.198	1.187 (0.845-6.775)	0.852	1.150 (0.912-1.136)	0.309
TNM	3.826 (1.034-5.082)	**0.000**	9.846 (0.364-20.682)	0.159	0.318 (0.116-1.863)	0.582
Tumor location	3.329 (0.782-5.552)	0.115	0.249 (0.036-0.905)	0.968	0.415 (0.051-3.915)	0.979
Intensity histone modification staining	0.249 (0.107-0.582)	**0.001**	0.067 (0.007-0.599)	**0.016**	1.069 (0.973-1.274)	0.235

*Survival analyses are shown for H3K27me3 and H3K9Ac staining intensity. The total number of patients included in the analyses was n=63 for H3K27me3 analyses and n=61 for H3K9Ac analyses, corresponding to LINE-1 low and high methylation categories, respectively. Hazard ratios are displayed for both Cox proportional hazard univariate and multivariate analyses and 95% confidence intervals are given for the histone modification intensity entered as a continuous variable. In multivariate analyses, age was entered as a continuous variable. For circumferential margin the 'negative' group was used as reference, for TNM stage I was used as reference. Tumor location is divided into the categories rectum, anal region, or other, using rectum as the reference category. Statistically ignificant values are indicated in **bold**.*

Both H3K27me3 (p=0.049) and H3K9Ac (p=0.14) show differences in overall survival between high and low intensity groups (Figures 4A and 4B, respectively). For H3K27me3, low intensity scores are correlated with worse overall survival, whereas for H3K9Ac, high intensity is correlated with worse overall survival. As for tumor recurrence, histone modification intensities were correlated with local recurrence with H3K27me3 (p=0.001) and H3K9Ac (p=0.084), but not with distant recurrence with H3K27me3 (p=0.172) and H3K9Ac (p=0.291). Multivariate analyses including LINE-1 methylation and histone modification intensities as continuous variables showed that LINE-1 methylation is a dominant factor in determining clinical outcome, as adding histone modification intensities did not improve the multivariate model. LINE-1

methylation showed correlation with overall survival (with addition of H3K27me3 p=0.062 and H3K9Ac p=0.15) and distant recurrence-free survival (with addition of H3K27me3 p=0.019 and H3K9Ac p=0.018), but not with local recurrence-free survival (with addition of H3K27me3 p=0.82 and H3K9Ac p=0.97).

In order to further study the relationship between LINE-1 methylation and histone modifications, we stratified histone modifications according to LINE-1 methylation levels. H3K27me3 intensity was found to significantly correlate with overall survival (p=0.020) and local recurrence-free survival (p=0.017) only when LINE-1 methylation was low (Figures 5A and 5C, respectively). Within this patient subset with low LINE-1 methylation, patients with high H3K27me3 intensity scores show better survival rates than patients with low presence of H3K27me3. High H3K27 methylation could have a "protective" function in the cells, preventing deregulated gene expression when DNA methylation is absent. H3K27me3 did not show any differences between high and low intensity groups when LINE-1 methylation was high, with overall survival (p=0.37) and local recurrence-free survival (p=0.28), again indicating that LINE-1 methylation is a dominant factor determining clinical outcome in this patient group. Uni- and multivariate analyses for H3K27me3 also showed statistically significant differences between high and low staining intensity groups when LINE-1 methylation was low, both for overall survival (univariate p=0.024, multivariate p=0.001) and for local recurrence-free survival (univariate p=0.046, multivariate p=0.016) (Table 3). Stratifying H3K27me3 intensity scores according to Alu methylation status did not show any significant differences (Figures 5B and 5D).

H3K9Ac intensity correlated with local recurrence-free survival (p=0.030) only when LINE-1 methylation was high (Figure 5E). The presence of H3K9 acetylation in combination with high

Figure 4
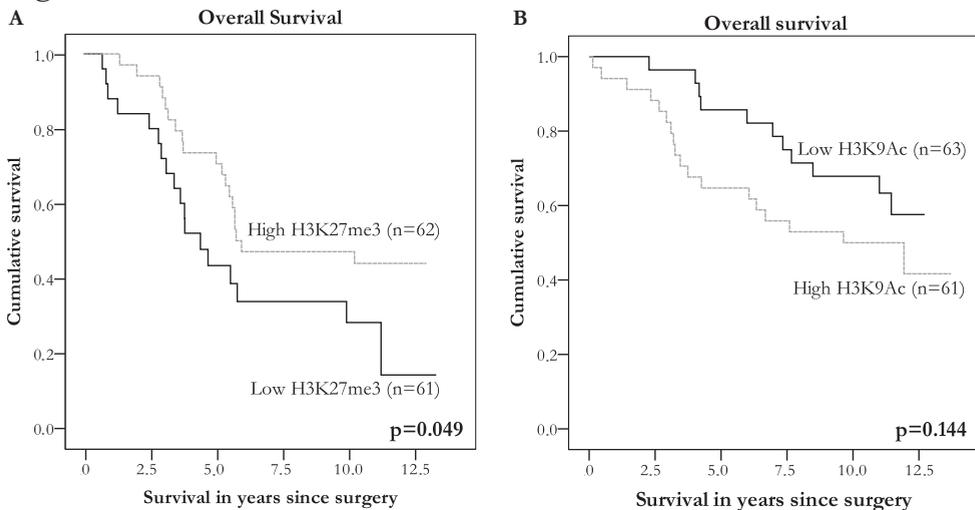
Survival curves of histone modifications H3K27me3 and H3K9Ac by expression levels. *Survival curves were made to visualize differences in survival and recurrence between patients with high and low expression of H3K27me3 or H3K9Ac. Log-rank tests were performed to calculate the difference between the low and high H3K27me3 expression groups. Survival times were calculated as time from surgery till an event (death).* **A.** *Cumulative incidence curves showing overall survival of the high and low methylation groups based on H3K27me3 expression.* **B.** *Cumulative incidence curves showing overall survival in the high and low methylation groups based on H3K9Ac expression.*

Figure 5

A

Overall survival - low LINE-1 methylation

p=0.020

High H3K27me3 (n=32)

Low H3K27me3 (n=31)

Cumulative survival

Survival in years since surgery

B

Overall survival - low Alu methylation

p=0.868

High H3K27me3 (n=32)

Low H3K27me3 (n=31)

Cumulative survival

Survival in years since surgery

C

Local recurrence-free survival - low LINE-1 methylation

p=0.017

Low H3K27me3 (n=31)

High H3K27me3 (n=32)

Cumulative incidence

Local recurrence-free interval (yrs)

D

Local recurrence-free survival - low Alu methylation

p=0.244

Low H3K27me3 (n=31)

High H3K27me3 (n=32)

Cumulative incidence

Local recurrence-free interval (yrs)

E

Local recurrence-free survival - high LINE-1 methylation

p=0.030

High H3K9Ac (n=32)

Low H3K9Ac (n=28)

Cumulative incidence

Local recurrence-free interval (yrs)

F

Local recurrence-free survival - high Alu methylation

p=0.563

High H3K9Ac (n=32)

Low H3K9Ac (n=28)

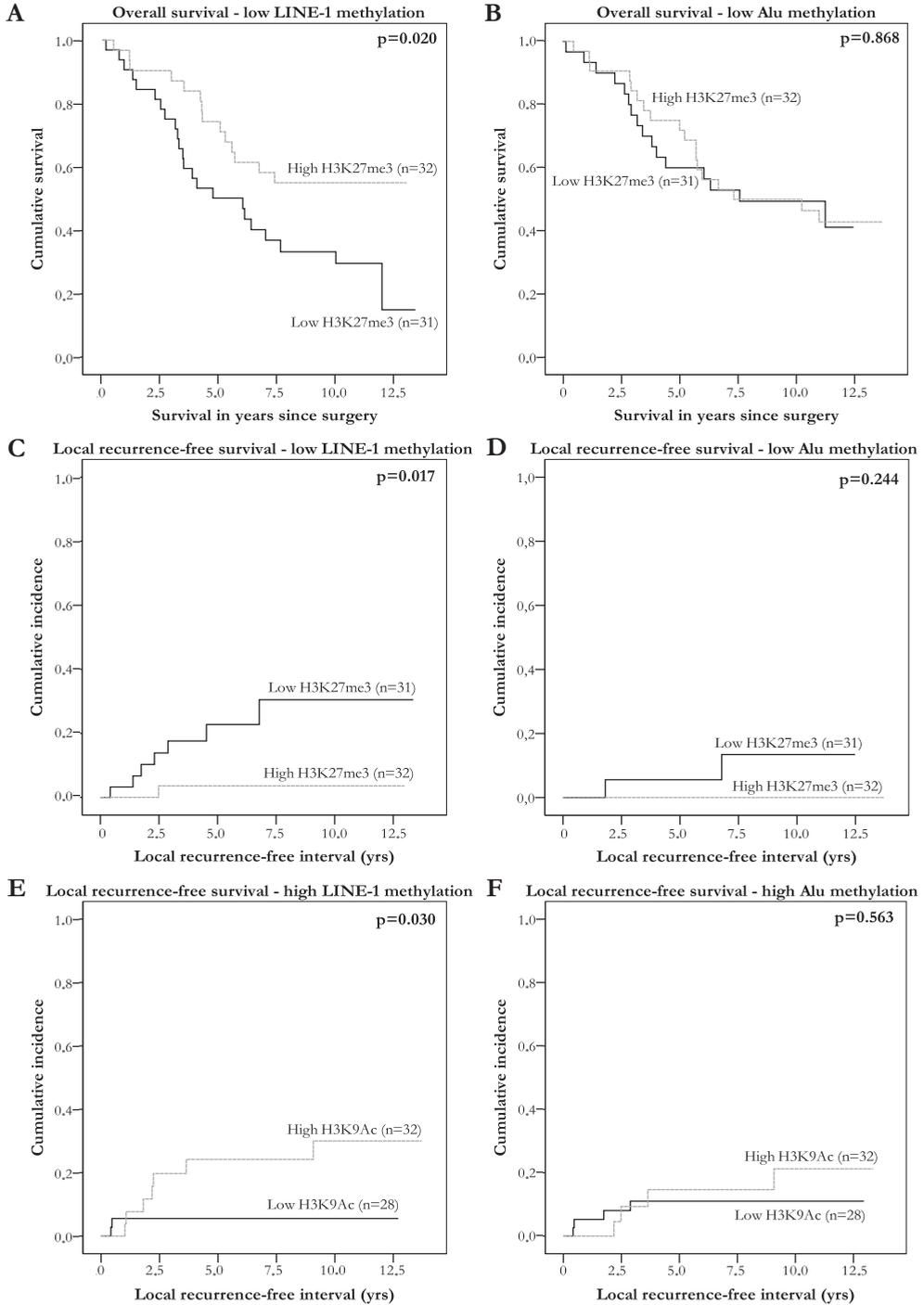Cumulative incidence

Local recurrence-free interval (yrs)

*Figure 5 (see previous page) Survival curves of histone modifications H3K27me3 and H3K9Ac according to LINE-1 or Alu methylation levels. Survival curves were made to visualize differences in survival and recurrence between patients with high and low expression of H3K27me3 and H3K9Ac. Log-rank tests were performed to calculate the difference between the low and high H3K27me3 or H3K9Ac expression groups, respectively. Survival times were calculated as time from surgery till an event (death or recurrence). **A**. Overall survival based on H3K27me3 expression in the LINE-1 methylation low group. **B**. Overall survival based on H3K27me3 expression in the Alu methylation low group. **C**. Local recurrence-free survival based on H3K27me3 expression in the LINE-1 methylation low group. **D**. Local recurrence-free survival based on H3K27me3 expression in the Alu methylation low group. **E**. Local recurrence-free survival based on H3K9Ac expression in the LINE-1 methylation high group. **F**. Local recurrence-free survival based on H3K9Ac expression in the Alu methylation high group.*

levels of DNA methylation could affect disease outcome through activation of aberrant gene expression. H3K9Ac did not show any differences between high and low intensity groups when LINE-1 methylation was low, with overall survival (p=0.83) and local recurrence-free survival (p=0.68). Univariate analyses showed a trend for H3K9Ac, when LINE-1 methylation is high, with local recurrence-free survival (p=0.066) (Table3). Unfortunately, in multivariate analyses, this trend was no longer observed. H3K9Ac did not show a significant correlation with overall survival in either uni- or multivariate analyses. Stratifying H3K9Ac intensity according to Alu methylation status did not show any significant differences (Figure 5F).

Discussion

The results presented in this paper showed that LINE-1 methylation should be regarded as an independent prognostic biomarker in early-stage rectal cancer. LINE-1 and Alu methylation were assessed using quantitative PCR after needle microdissection of tumor tissue areas, which were carefully marked by an experienced pathologist. Tumor tissue extracted using needle microdissection, in contrast to laser capture microdissection, may include more stromal tissue, including inflammatory cells. We therefore selected only tumor areas with >80% tumor epithelial cells, thereby minimizing the amount of stroma included in these assays. Although previous reports showed an association of lymphocytic infiltrates with clinical outcome (29) in colorectal cancer, in our rectal cancer study cohort we did not find a correlation between the amount of lymphocytic infiltrate and LINE-1 methylation that could influence our analyses. Therefore, it is not likely that our findings are influenced by stroma or infiltrating lymphocytes. In addition, Irahara *et al.* have shown, by direct comparison, that LINE-1 methylation levels are comparable between needle microdissected and laser capture microdissected tumor tissues, indicating that the effect of contaminating cells on LINE-1 methylation is indeed minimal (30). BRAF mutation and MSI status were not determined in this study cohort, as the prevalence of BRAF mutations (around 2%; 31,32) and MSI (around 2%; 33) is very low in rectal cancer. The impact of these markers on clinical outcome in multivariate analyses would therefore be negligible.

Because we were looking for a rapid, robust, high-throughput and highly sensitive assay, we chose to use a quantitative real-time PCR assay instead of pyrosequencing to measure LINE-1 methylation. Pyrosequencing relies on PCR amplification of the region of interest, followed by sequencing of this PCR-amplified product. According to Nelson *et al.* (34) and Irahara *et al.* (30) pyrosequencing of LINE-1 sequences typically includes 3-6 CpG sites at the 5' end of LINE-1 sequences, which is comparable to the quantitative real-time PCR assay conducted in this study. Also, as suggested in Figure 1 of the pyrosequencing analysis by Irahara *et al.*, the methylation percentages of the individual CpG sites measured with the LINE-1 pyrosequencing assay seem

very similar within one sample. Therefore, measuring the three CpG sites at once using the qPCR method, instead of measuring individual CpG sites by pyrosequencing, is representative. In addition, for survival analyses conducted in this study, the median methylation percentage was used to divide the patients into high- and low-risk groups. As described by Irahara *et al.*, the average of the relative amounts of methylated cytosines found using pyrosequencing was used as overall LINE-1 methylation level in each sample, which is a similar approach as we used with our qPCR data. Pyrosequencing information would probably result in a lower median methylation percentage, as hemimethylated CpGs can be accurately measured. However, this would yield a lower LINE-1 methylation percentage for every patient in our cohort and we expect this will not affect the distribution of the patients into the two groups based on the median LINE-1 methylation percentage. Therefore, we are confident the qPCR method used in this study was a valid method to study LINE-1 methylation.

In our study cohort, stage III and IV patients did not show a significant correlation of LINE-1 methylation with survival and recurrence, most likely because other clinical factors such as nodal status and circumferential resection margin involvement have a significant role in advanced stage cancers, overpowering the effect of an earlier event in tumorigenesis. Epigenomic aberrations in tumors of stage I and II patients without invasive tumor characteristics, however, may be associated with the development of aggressive tumors and have clinical relevance for risk stratification. LINE-1 methylation has been described as an early-stage tumor marker in previous studies (18,35).

Methylation of LINE-1 and Alu repeats have both been used as surrogate markers for genome-wide methylation status (9). Based on the general hypothesis that tumorigenesis is associated with genome-wide DNA hypomethylation and locus-specific hypermethylation of individual CpG islands (15,36), we expected to see a decrease in both Alu and LINE-1 methylation levels with increasing TNM tumor stage in rectal cancer tissues. However, as we showed here that Alu methylation, in contrast with LINE-1, did not change with tumor progression and that methylation levels of LINE-1 and Alu did not correlate, our results suggest a more specific effect of LINE-1 methylation in early rectal tumors instead of reflecting a genome-wide methylation status. Other studies also suggest a specific role for LINE-1 methylation in tumorigenesis, while other repetitive sequences such as Alu repeats remain equally methylated (37). Active LINE-1 sequences, an estimated 30-60 copies per cell, have retrotranspositional activity (38) and can relocate to other (non-)coding regions (7,13,39), thereby contributing to (epi)genetic variation (40,41). In normal cells, retrotransposition of repeat sequence elements is repressed by methylation of cytosine residues (42). Reintegration of LINE-1 retrotransposons can disrupt genes and regulate their expression (43,44), as described for APC in colon cancer (16). Using the genome vicinity information of the full-length active LINE-1 sequences provided in the L1base database (Max-Planck Institute Berlin and University of Würzburg; 45), we found that several LINE-1 sequences are located near or in (intronic) gene regions. As neighbouring DNA methylation patterns can influence nearby promoter regions (46,47) or longer stretches of DNA, up to 2kb distant (48), demethylation of LINE-1 sequences might therefore represent demethylation events of the coding gene promoters in their vicinity and hence influence expression of genes in these regions, translating into worse clinical outcome for the patient. This hypothesis was further supported by Hur *et al.*, who show that specific LINE-1 sequences residing in intronic regions of several proto-oncogenes were hypomethylated in metastatic tissues compared to the

corresponding primary tumors (49), indicating that differentially methylated LINE-1 sequences can indeed influence progression of colorectal tumors.

Recent articles by Goel *et al.* and Ogino *et al.* describe that LINE-1 hypomethylation is correlated with shorter survival, independent of clinical or molecular features, in non-MSI (microsatellite instability) familial tumors (50,51). Mechanisms predisposing patients with a family history of (and thus early-onset) colorectal cancer, but with no evidence of MSI or other molecular features, for the development of a tumor are still unknown. The authors of both articles conclude that LINE-1 might be an important factor promoting tumor growth in familial cases of (MSS) colorectal cancers. In our study, consisting of MSS rectal cancer patients, we also show a significant prognostic role for LINE-1 hypomethylation in determining clinical outcome. Together, these studies suggest that LINE-1 methylation might represent a frequently deregulated regulatory epigenetic mechanism that might promote primary tumor progression and metastasis of rectal tumors. Hence, LINE-1 methylation may potentially serve as a new biomarker to identify high-risk patients. Future studies will have to be performed to validate the clinical utility of LINE-1 in rectal cancer.

The histone modifications reported in this study support the clinical impact of genome-wide changes in epigenetic modifications in rectal cancer patients. On the basis of our current knowledge about the interplay between histone modifications and DNA methylation, we hypothesized that silencing modification H3K27me3 should follow the same pattern as LINE-1 methylation, as both epigenetic mechanisms are involved in silencing of gene expression. Indeed, low expression of H3K27me3 was associated with worse survival. The finding that H3K27me3 was associated with clinical outcome only when LINE-1 methylation is low, supports the generally accepted hypothesis that DNA methylation and histone modifications together control gene expression and thereby define the cellular phenotype. Activating histone modification H3K9Ac was expected to show opposite results, as the presence of this histone modification will lead to (aberrant) activation of gene expression. Indeed, high expression of H3K9Ac was associated with a shorter survival and higher chance of recurrence compared to patients with low H3K9Ac expression. As both H3K27me3 and H3K9Ac expression only correlate with LINE-1 methylation and not with Alu methylation, we again conclude that LINE-1 methylation may be involved in specific tumor progression events in rectal cancer, rather than reflecting genome-wide methylation status in these tumors. Histone modifications H3K27me3 and H3K9Ac intensity scores can further subdivide rectal cancer patients into high- or low risk groups when stratified according to LINE-1 methylation status. This indicates that LINE-1 methylation and histone modifications work closely together in determining gene expression and hence tumor progression and ultimately clinical outcome.

In conclusion, we have shown in this study that LINE-1 methylation is an independent predictor of survival and recurrence in early-stage rectal cancer. Expression of histone modifications H3K9Ac and H3K27me3 further supports an active role in rectal cancer progression of LINE-1. Further research should be conducted to investigate the exact function of LINE-1 elements, and their influence on neighbouring genes, in order to better understand the complex nature of the rectal tumorigenic process. We show here that methylation of LINE-1 repeat sequences can be used as a biomarker to distinguish rectal cancer patients with a high risk of distant recurrence at an early stage, and therefore, has potential to complement the current TNM staging system.

Acknowledgements

References

(1) Edge SB. AJCC Cancer Staging Manual, 7th edition. 7th ed. Springer: New York, 2010.

(2) Zeestraten EC, Kuppen PJ, van de Velde CJ et al. Prediction in rectal cancer. Semin Radiat Oncol 2012; 22(2):175-83.

(3) Lao VV, Grady WM. Epigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol 2011; 8(12):686-700.

(4) de Maat MF, Umetani N, Sunami E et al. Assessment of methylation events during colorectal tumor progression by absolute quantitative analysis of methylated alleles. Mol Cancer Res 2007; 5(5):461-71.

(5) de Maat MF, van de Velde CJ, van der Werff MP et al. Quantitative analysis of methylation of genomic loci in early-stage rectal cancer predicts distant recurrence. J Clin Oncol 2008; 26(14):2327-35.

(6) de Maat MF, van de Velde CJ, Benard A et al. Identification of a quantitative MINT locus methylation profile predicting local regional recurrence of rectal cancer. Clin Cancer Res 2010; 16(10):2811-8.

(7) Sellis D, Provata A, Almirantis Y. Alu and LINE1 distributions in the human chromosomes: evidence of global genomic organization expressed in the form of power laws. Mol Biol Evol 2007; 24(11):2385-99.

(8) Lander ES, Linton LM, Birren B et al. Initial sequencing and analysis of the human genome. Nature 2001; 409(6822):860-921.

(9) Weisenberger DJ, Campan M, Long TI et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005; 33(21):6823-36.

(10) Burwinkel B, Kilimann MW. Unequal homologous recombination between LINE-1 elements as a mutational mechanism in human genetic disease. J Mol Biol 1998; 277(3):513-7.

(11) Kazazian HH, Jr., Wong C, Youssoufian H et al. Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. Nature 1988; 332(6160):164-6.

(12) Bae JM, Shin SH, Kwon HJ et al. ALU and LINE-1 hypomethylations in multistep gastric carcinogenesis and their prognostic implications. Int J Cancer 2011; 131(6):1323-31.

(13) Beck CR, Garcia-Perez JL, Badge RM et al. LINE-1 elements in structural variation and disease. Annu Rev Genomics Hum Genet 2011; 12:187-215.

(14) Chen JM, Stenson PD, Cooper DN et al. A systematic analysis of LINE-1 endonuclease-dependent retrotranspositional events causing human genetic disease. Hum Genet 2005; 117(5):411-27.

(15) Cho NY, Kim BH, Choi M et al. Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. J Pathol 2007; 211(3):269-77.

(16) Miki Y, Nishisho I, Horii A et al. Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. Cancer Res 1992; 52(3):643-5.

(17) Sunami E, Vu AT, Nguyen SL et al. Quantification of LINE1 in circulating DNA as a molecular biomarker of breast cancer. Ann N Y Acad Sci 2008; 1137:171-4.

(18) Sunami E, de MM, Vu A et al. LINE-1 hypomethylation during primary colon cancer progression. PLoS One 2011; 6(4):e18884.

(19) Rodenhiser D, Mann M. Epigenetics and human disease: translating basic biology into clinical applications. CMAJ 2006; 174(3):341-8.

(20) van Engeland M., Derks S, Smits KM et al. Colorectal cancer epigenetics: complex simplicity. J Clin Oncol 2011; 29(10):1382-91.

(21) Klein Kranenbarg E., van de Velde CJ. Surgical trials in oncology. the importance of quality control in the TME trial. Eur J Cancer 2002; 38(7):937-42.

(22) McShane LM, Altman DG, Sauerbrei W et al. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 2005; 93(4):387-91.

(23) Tanemura A, Terando AM, Sim MS et al. CpG island methylator phenotype predicts progression of malignant melanoma. Clin Cancer Res 2009; 15(5):1801-7.

(24) Sunami E, de MM, Vu A et al. LINE-1 hypomethylation during primary colon cancer progression. PLoS One 2011; 6(4):e18884.

(25) Umetani N, Giuliano AE, Hiramatsu SH et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. J Clin Oncol 2006; 24(26):4270-6.

(26) Bustin SA, Benes V, Garson JA et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009; 55(4):611-22.

(27) Umetani N, Kim J, Hiramatsu S et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. Clin Chem 2006; 52(6):1062-9.

(28) van Nes JG, de Kruijf EM, Faratian D et al. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res Treat 2011; 125(3):671-85.

(29) Nagtegaal ID, Marijnen CA, Kranenbarg EK et al. Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. BMC Cancer 2001; 1:7.

(30) Irahara N, Nosho K, Baba Y et al. Precision of pyrosequencing assay to measure LINE-1 methylation in colon cancer, normal colonic mucosa, and peripheral blood cells. J Mol Diagn 2010; 12(2):177-83.

(31) Derbel O, Wang Q, Desseigne F et al. Impact of KRAS, BRAF and PI3KCA mutations in rectal carcinomas treated with neoadjuvant radiochemotherapy and surgery. BMC Cancer 2013; 13:200.

(32) Gaedcke J, Grade M, Jung K et al. KRAS and BRAF mutations in patients with rectal cancer treated with preoperative chemoradiotherapy. Radiother Oncol 2010; 94(1):76-81.

(33) Slattery ML, Curtin K, Wolff RK et al. A comparison of colon and rectal somatic DNA alterations. Dis Colon Rectum 2009; 52(7):1304-11.

(34) Nelson HH, Marsit CJ, Kelsey KT. Global methylation in exposure biology and translational medical science. Environ Health Perspect 2011; 119(11):1528-33.

(35) Murata A, Baba Y, Watanabe M et al. Methylation levels of LINE-1 in primary lesion and matched metastatic lesions of colorectal cancer. Br J Cancer 2013; 109(2):408-15.

(36) Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007; 8(4):286-98.

(37) Perrin D, Ballestar E, Fraga MF et al. Specific hypermethylation of LINE-1 elements during abnormal overgrowth and differentiation of human placenta. Oncogene 2007; 26(17):2518-24.

(38) Sassaman DM, Dombroski BA, Moran JV et al. Many human L1 elements are capable of retrotransposition. Nat Genet 1997; 16(1):37-43.

(39) Beck CR, Collier P, Macfarlane C et al. LINE-1 retrotransposition activity in human genomes. Cell 2010; 141(7):1159-70.

(40) de Andrade A., Wang M, Bonaldo MF et al. Genetic and epigenetic variations contributed by Alu retrotransposition. BMC Genomics 2011; 12:617.

(41) Ogino S, Nosho K, Kirkner GJ et al. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. J Natl Cancer Inst 2008; 100(23):1734-8.

(42) Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 1997; 13(8):335-40.

(43) Chow JC, Ciaudo C, Fazzari MJ et al. LINE-1 activity in facultative heterochromatin formation during X chromosome inactivation. Cell 2010; 141(6):956-69.

(44) Matlik K, Redik K, Speek M. L1 antisense promoter drives tissue-specific transcription of human genes. J Biomed Biotechnol 2006; 2006(1):71753.

(45) Penzkofer T, Dandekar T, Zemojtel T. L1Base: from functional annotation to prediction of active LINE-1 elements. Nucleic Acids Res 2005; 33(Database issue):D498-D500.

(46) Wang X, Fan J, Liu D et al. Spreading of Alu methylation to the promoter of the MLH1 gene in gastrointestinal cancer. PLoS One 2011; 6(10):e25913.

(47) Zhang Y, Shu J, Si J et al. Repetitive elements and enforced transcriptional repression co-operate to enhance DNA methylation spreading into a promoter CpG-island. Nucleic Acids Res 2012; 40(15):7257-68.

(48) Irizarry RA, Ladd-Acosta C, Wen B et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 2009; 41(2):178-86.

(49) Hur K, Cejas P, Feliu J et al. Hypomethylation of long interspersed nuclear element-1 (LINE-1) leads to activation of proto-oncogenes in human colorectal cancer metastasis. Gut 2013; 63(4):635-46.

(50) Goel A, Xicola RM, Nguyen TP et al. Aberrant DNA methylation in hereditary nonpolyposis colorectal cancer without mismatch repair deficiency. Gastroenterology 2010; 138(5):1854-62.

(51) Ogino S, Nishihara R, Lochhead P et al. Prospective study of family history and colorectal cancer risk by tumor LINE-1 methylation level. J Natl Cancer Inst 2013; 105(2):130-40.

2