CHAPTER 4

LINE-1 methylation analysis in colorectal cancer shows progression of hypomethylation at early stage

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Abstract

Background: Methylation levels of DNA repeats such as long interspersed nucleotide ele-
ments (LINE-1) are representative of global methylation status and play an important role in maintenance of genomic stability. LINE-1 methylation is known to be disrupted in many types of cancer. In this study we report on high detail analyses of LINE-1 methylation levels at different stages of colorectal cancer (CRC) development and at different stage of CRC disease.

Material and Methods: Sample DNA was collected by laser-capture microdissection (LCM) treated by a newly introduced protocol of on-cap \textit{(in situ)} DNA sodium bisulfite modification (SBM). LINE-1 hypomethylation index (LHI), a higher index indicates more hypomethylation, was measured by absolute quantitative analysis of methylated alleles (AQAMA).

Results: LHI was significantly higher in adenoma tissue (n=25) compared to normal epi-
thelium (n=23, P=0.0003) and cancer mesenchymal tissue (n=22, P<0.0001). LHI did not differ significantly between adenoma and cancer tissue (n=24, P=0.20). No significant differ-
ences were found in LHI between normal epithelium from non-cancer (n=13), adeno-
ma (n=18) and cancer patients. Tumor heterogeneity was tested and LHI did not differ sig-
nificantly comparing cells collected from a tumor’s luminal side compared with cells from the invasive margin. LHI increased with higher T-stage (P<0.04) and was significantly hig-
her in node-positive versus node-negative disease (P=0.03) and was significantly higher in stage IV versus all other disease stages (P<0.05).

Conclusion: By using quantitative LINE-1 analysis with on-cap SBM microdissection tech-
niques this is the first study to demonstrate hypomethylation of LINE-1 to increase with advanced disease stage in colorectal cancer.
**Introduction**

LINE-1 stands for long interspersed nucleotide elements and constitutes about 17-18% of the human genome. Roughly 500,000 truncated and 3000 to 5000 full length LINE-1s are dispersed throughout the human genome. LINE-1 sequences are moderately CpG rich and most CpG’s locate in the 5’ region that can behave as a internal promoter. In normal somatic cells, LINE-1s are heavily methylated restricting activities of retrotransposal elements and genomic instability.

Numerous studies have been reported identifying colorectal cancer (CRC) cancer-associated epigenetic aberrations such as promoter region hypermethylation of tumor suppressor genes. Another characteristic alteration in CRC regarding DNA methylation in cancer is global hypomethylation. LINE-1 methylation status is thought to represent the genome-wide DNA methylation status such as global hypomethylation, since LINE-1 sequences are highly repeated and widely interspersed human retrotransposons. The role of global DNA hypomethylation in cancer is unclear, although it may cause genomic instability and correlates with tumor progression and invasiveness in several types of cancer including colon cancer. Regarding colon cancer, Bariol et al. revealed that the global DNA methylation level was higher in neoplastic lesions (including hyperplastic polyps and adenoma) than in normal mucosa. Ogino et al. suggests that LINE-1 hypomethylation status of colon cancer associates with poor prognosis. The progression of LINE-1 hypomethylation level according to the sequence of colorectal tumor disease stage remains unclear to date.

Cancer tissue is highly heterogeneous and contains various cells, such as normal (i.e. stromal cells and infiltrating lymphocytes) and malignant cells and their distribution differs in each cancer patient. This heterogeneous nature of cancer tissue may confound molecular analysis, especially in case of a molecular target such as LINE-1 methylation analysis, because unmethylated LINE-1 is non-cancer-specific. In this study we therefore utilize automated laser-capture microdissection (LCM) to harvest the cells of interest directly without contamination of non-cancer cells. A precise quantification method for analysis of methylation status is further necessary to evaluate the degree of LINE-1 methylation. Absolute quantitative assessment of methylated alleles (AQAMA) assays was used for this purpose in this study. As previously demonstrated, the capacity of AQAMA to discern differences in methylation level was excellent. The single reaction AQAMA assay provides good control because results can be analyzed without the need to compensate for the variability of two or three separate PCR reactions with different setting and reaction kinetics. The approach allows accurate derived analysis of histopathology microdissected cells from PE tissue sections.

Sodium bisulfite modification (SBM) of genomic DNA is commonly used method to discern methylation status of CpG islands. Sodium bisulfite converts cytosines but not 5-methylcytosines to uracil and the changes in sequence of the DNA after SBM can be analyzed by many kinds of methylation assays including AQAMA assay. Conventional SBM results into 84% to 96% loss of sample DNA. This significant loss of template DNA causes the necessity for a high volume of start material tissue samples and high number of cycles for PCR. To reduce the loss of sample DNA from cell collected by LCM we developed on-cap SBM. On-cap SBM was designed to minimize DNA loss by eliminating DNA purification steps. In the process of on-cap SBM, DNA is modified in-situ while the cells are...
still attached on the cap which is used for LCM. DNA is collected afterwards and processed for AQAMA. We have developed and optimized a protocol for on-cap SBM in this study by which sufficient DNA conversion rates can be achieved and relatively low volumes of tissue sample are needed for the subsequent AQAMA LINE-1 hypomethylation assay.

LCM, on-cap SBM and AQAMA was combined and used in samples from patients with adenomas containing invasive adenocarcinoma and patients with advanced colon cancer. This allowed to analyze the development of LINE-1 hypomethylation levels during tumor progression and differences of LINE-1 hypomethylation levels between node positive and node negative colon cancer patients. Our results indicate that LINE-1 hypomethylation occurs in the early stage of colorectal tumorigenesis such as adenoma and proceeds according to the tumor stage such as depth of invasion and establishment of lymph node metastasis.

Materials and Methods

Measurement of LINE1 hypomethylation

The methylation status of LINE1 was evaluated by absolute quantitative assessment of methylation alleles (AQAMA) assay to make accurate assessment of CpG island methylation levels\(^9\). Before performing the AQAMA assay of LINE1 methylation status, sodium bisulfite modification (SBM) was applied on each DNA sample as previously described\(^{12}\). AQAMA requires one forward and one reverse primer which will amplify the target sequence independent from the methylation status, as those forward and reverse primer set does not contain any CpG. The methylation status is assessed by two minor-groove-binding (MGB) molecule containing probes (Applied Biosystems): one methylation specific and one unmethylation specific. The 5' primer, 3' primer, methylation-specific probe and unmethylation-specific probe are listed as follows: 5'-GGGTTTATTTTATTAGGGAGTGTTAGA-3' (forward), 5'-TCACCCCTTTCTTTAACTCAAA-3' (reverse), FAM-5'-TGCGCGAGTCGAATGAGTGGAC-3'-MGB-BHQ and VIC-5'-TGTGTGAGTTGAAGTAGGG-3'-MGB-BHQ. The reaction mixture for each AQAMA PCR consisted of DNA template, 0.4 μmol/L of the forward and reverse primer, 1.4 unit of iTaq DNA polymerase (Bio-Rad, Hercules, CA, USA), 350 μmol/L of each deoxynucleotide triphosphate and 0.025 pmol of each MGB probe with 5 mmol/L Mg\(^{2+}\). PCR amplification was performed with pre-cycle heat activation of DNA polymerase at 95° C for 10 minutes, followed by 40 cycles of denaturation at 95° C for 15 seconds, annealing and extension at 60° C for 60 seconds. The absolute copy number in each sample was determined using a standard curve established by amplifying six aliquot duplicates of templates with known copy numbers (10\(^{-6}\) to 10\(^{-1}\) copies). All quantitative PCR assays were performed in a blinded fashion without knowledge of specimen identity. Mean values were calculated from triplicate reactions. LINE1 hypomethylation index (LHI) of each sample was calculated as follows: LINE1 LHI = unmethylated copy number / (methylated copy number + unmethylated copy number).

On-cap SBM assay optimization

To measure LINE1 hypomethylation index after micro-dissection using LCM, we develo-
ped the on-cap SBM procedure based on previously introduced on-slide SBM technique. On-cap SBM was designed to analyze methylation status of genes in small amount of DNA obtained from formalin fixed paraffin embedded archival tissue (FF-PEAT). On-cap SBM aims to eliminate DNA isolation and purification steps that are performed before the actual SBM in the classic procedure which is the main cause of DNA loss. On-cap SBM contains three steps; denaturing of DNA, SBM and collection of modified DNA. First, the cells micro-dissected from deparaffinized and rehydrated tissue sections that stick on the cap used in LCM are incubated in 0.2 mol/L NaOH at room temperature for 15 min. Then the samples on the cap are incubated in 3 mol/L sodium bisulfite solution with 0.5 mmol/L hydroquinone (pH 5) in the dark. Three incubation setting were tested regarding the conversion rates (the rate of modification of cytosine to uracil) and yields of modified DNA and incubation setting of 60 degrees for 8 hours was chosen as optimal (see results section). After incubation, samples on the cap were rinsed with distilled water, soaked in 0.3 mol/L NaOH for 15 minutes to desulfonate the modified cytosines, and then desalted in distilled water at 60°C for 2 hours. Finally, 50 μL lysis buffer containing 4 μg proteinase K, 2.5% Tween 20, 50 mmol/L Tris, and 1 mmol/L EDTA were added on the cap and incubated at 50°C for 24 hours followed by heat deactivation of proteinase K at 95°C for 10 minutes. In each subsequent AQAMA reaction, 1-2 μL lysate was used as a template without DNA purification.

**FF-PEAT colorectal tumor samples**

FF-PEAT samples of colorectal cancers were obtained from patients who underwent colectomy or proctectomy between 1995 and 1998 at Saint John's Health Center (Santa Monica, CA) and consented for research use of their tissue specimens according to the guidelines of Saint John's Health Center/John Wayne Cancer Institute's (Santa Monica, CA) review board. All tissue specimens had been fixed in 10% buffered formalin for 24 hours and paraffin embedded. For LCM followed by on-cap SBM and LINE1 methylation analysis by AQAMA, sections (4 μm) were cut with a microtome from each FF-PEAT block.

To assess the differences of LINE-1 methylation status between normal mucosa, adenoma, cancer and cancer mesenchymal connective tissue, 25 samples of early CRC in adenoma were selected by a pathologist specialized in colorectal cancer that contained all of these four tissue categories. To study malignant alteration, 92 surgically resected specimen CRC were collected to investigate the alteration of LINE1 methylation status in accordance with cancer progression. The correlation between clinico-pathological stage such as T stage, nodal status and dukes stage and LINE-1 methylation index were analyzed.

**Statistical analysis**

All data are expressed as mean+/-S.D., and percentages as appropriate. Mean value of LHI between two groups such as adenoma and early cancer, node negative and node positive were compared using Student's t-test. All statistical analysis including receiver operating characteristic (ROC) curve analysis were performed using JMP 8 (SAS Institute, Cary, NC) and a P-value less than 0.05 (two-sided) was considered significant.
Results

AQAMA linearity for LINE-1 methylation level assessment

First, we evaluated the accuracy of AQAMA in assessing various levels of LINE-1 methylation. For this study we prepared stepwise mixtures of the methylated and unmethylated cDNA standard and measured them as samples with unknown methylation level. The linearity of AQAMA assay for LINE-1 methylation level (figure 1) was evaluated by Pearson's coefficient of linearity: 0.9897 (P<0.001). This results shows that the AQAMA LINE-1 assay can discern 10% methylation level differences adequately.

![Figure 1. Graph showing linearity of the AQAMA LINE-1 methylation assay to measure 10% differences in sample hypomethylation levels.](image)

Optimization of on-cap SBM settings

Automated LCM was performed to harvest cells of interest (figure 2a) to establish how many cells are needed for successful AQAMA measurements. Three different sample volumes from tissue areas of $10^4$, $10^5$ and $10^6 \mu m^2$ harvested from 7μm thickness formalin fixed paraffin embedded archived tissue (FF-PEAT) were tested. AQAMA measures copy numbers and we established that $2 \times 10^5 \mu m^2$ of 7μm thickness FF-PEAT results in about $1 \times 10^5$ copy numbers of modified LINE-1 DNA and this provides clearly detectable fluorescent probe amplification signals for the LINE-1 methylation assay. After LCM, on-cap SBM was optimized in a similar manner as was performed in the reported studies introducing on-slide SBM\textsuperscript{13}. The conversion rate and yield were evaluated using eight tissue samples by assessing the copy number of modified and unmodified ALU repeat sequences, as previou-
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The conversion rates of modified DNA by on-cap SBM were 18.4±14.9%, 87.8±7.8% and 94.4±2.1% at incubation setting of 60º C for 2, 4 and 8 hrs, respectively (figure 2b). The conversion rates increased with increasing duration of incubation and after 8 hours it reached about 95%. The yield of total DNA (modified and unmodified DNA) did not decrease with increasing incubation time up to 8 (figure 2c). From these results, incubation setting of 60 for 8 hrs was chosen as optimal for on-cap SBM.

LINE-1 hypomethylation levels in colorectal tumor progression and cancer mesenchyme.

We have selected 25 patients with colorectal cancer in adenoma. Using LCM, four different tissue samples (normal mucosa, adenoma, cancer and cancer mesenchymal tissue) were collected from each patient. On-cap SBM and LINE-1 AQAMA assay was performed on each sample. Level of LINE-1 hypomethylation (LHI) were calculated as $Q_{\text{unmeth}} / (Q_{\text{unmeth}} + Q_{\text{meth}})$, where $Q_{\text{unmeth}}$ and $Q_{\text{meth}}$ are the absolute copy numbers of unmethylated DNA.
ted and methylated LINE-1, respectively. In normal mucosa adjacent to tumor lesions and cancer mesenchyme, the average LHI was 0.382 and 0.366, respectively. There was no difference of LHI between cancer mesenchyme and normal mucosa adjacent to tumor. LHI was significantly higher in adenoma part and cancer part than in normal mucosa (mean LHI=0.486, 0.523 and 0.382, respectively) (figure 3). From this we concluded that LINE-1 hypomethylation occurs in the early period of colorectal tumorigenesis. Further, these experiments demonstrated the necessity of using detailed procedures for sample DNA collection when analyzing LINE-1 methylation as methylation levels of cancer mesenchyme varied widely and are therefore likely to influence results when LCM is not used.

**Figure 3.** LHI assessment in normal large bowel epithelium, adenomatous cells, cancer cells and CRC mesenchymal cells.

**LINE-1 hypomethylation tissue heterogeneity**

To test whether there is heterogeneity of LINE-1 hypomethylation within a tumor, twenty T3N0 tumors were selected. Sample DNA was collected from the luminal surface and the deepest invasional site of each tumor. LHI of those two loci were compared (figure 4). The mean LHI was 0.58 in the surface and 0.54 in the deepest loci, respectively. LHIs were similar in the surface and in the deepest loci and tumor heterogeneity of LINE-1 hypomethylation could not be observed.
LINE-1 hypomethylation and CRC stage

To assess the alteration of LINE-1 hypomethylation level according to tumor progression, the correlation between Dukes stage and LHI was analyzed. Duke A samples (n=38), Dukes B (n=18) and Dukes C (n=29) samples were selected (figure 5a). The mean LHI in Dukes A, B and C was 0.533, 0.607 and 0.621, respectively. Dukes B and C tumors showed significantly higher levels of LINE-1 hypomethylation than Dukes A tumors. T1 (n=24), T2 (n=21), T3 (n=21) and T4 (n=26) cases were assessed, in which 35 positive lymph node cases were included. The increase of the LINE-1 hypomethylation level according to tumor invasion (figure 5b) and the difference of LINE-1 hypomethylation between lymph node positive and negative cases were assessed (figure 5c). The mean LHI of T1, T2, T3 and T4 was 0.50, 0.58, 0.60 and 0.65, respectively. Cases with positive lymph nodes showed significantly ($P=0.03$) more LINE-1 hypomethylation (LHI=0.64) than cases with negative lymph nodes (LHI=0.54). T stage advancement correlated significantly with increasing LHI ($p=0.01$). LINE-1 hypomethylation levels differed significantly between node positive and node negative cancers and the AUC was 0.69 to predict node positive cancer from whole samples according to ROC curve analysis (figure 5d).
Discussion

The alteration of DNA methylation patterns has been studied in many types of cancer\(^\text{14}\). Neoplasia is now epigenetically characterized by hypermethylation of specific cancer associated gene promoter regions and global DNA hypomethylation\(^\text{15, 16}\). LINE-1 constitutes about 17-18% of the human genome and about 500,000 truncated and 3000-5000 full length LINE-1s are dispersed throughout the human genome\(^\text{1, 17}\). LINE-1 sequences are moderately CpG rich and most CpG locate in the 5' region that can behave as an internal promoter. Therefore LINE-1 hypomethylation has been used as a surrogate for global DNA hypomethylation. LINE-1 hypomethylation in colorectal cancer has been described in several aspects; hypomethylation in cancer and adjacent normal mucosa, inverse association with microsatellite instability and association with poor prognosis\(^\text{8, 18-20}\).

Figure 5. Measured LHI values comparisons between clinical disease stage classifications. In A, B and C differences are shown between Duke stage, T-stage and N-stage, respectively. In D, a receiver-operator-curve (ROC) analysis is given for prediction of lymph node status by primary tumor LINE-1 hypomethylation analysis with an area under the curve (AUC) of 0.69.)
LINE-1 hypomethylation is not specific for cancer cells and about one third of LINE-1 is unmethylated in normal mucosa and cancer mesenchymal cells\textsuperscript{21, 22}. The possible contamination of sample DNA considered to be cancer with mesenchymal cells such as fibroblasts or lymphocytes may therefore cause inaccurate assessment. For this reason, especially regarding LINE-1 methylation analysis, isolation of target cells is necessary. To minimize the contamination and obtain to cells of interest only, we successfully integrated automated LCM in LINE-1 methylation analyses this study which has not been reported to date. We introduce on-cap SBM as a novel approach which makes DNA methylation assay efficient and enables evaluation of cells from small areas of target regions.

On-cap SBM was designed based on on-slide SBM\textsuperscript{13}. On-slide SBM is a powerful procedure for the methylation analysis using FF-PEAT, however, after on-slide SBM the specimen becomes too adhesive to the glass slide making efficient LCM to capture target cells difficult at times. On-cap SBM eliminates the numerous DNA isolation and purification steps of classic SBM which are the main cause of DNA loss dealing with small amounts of DNA such as isolated by LCM or from serum. We performed optimization studies and an 8 hour incubation showed excellent DNA conversion rates of approximately 95% was achieved and this relatively long incubation duration did not result into loss of converted DNA.

Combining automated LCM, on-cap SBM and AQAMA assay, LINE-1 hypomethylation was demonstrated in adenoma, an early stage of colorectal tumorigenesis, which is in agreement with previous studies\textsuperscript{8, 20}. Furthermore, the level of hypomethylation increased according to tumor stage. In previous studies, no correlation between tumor stage in colorectal cancer and LINE-1 hypomethylation level has been reported. One of the reasons for those differences may be the degree of mesenchymal cell DNA contamination to the sample DNA. Our results demonstrate that not only adjacent normal mucosa but also mesenchymal cells in cancer shows about one third loss of LINE-1 hypomethylation compared to normal epithelium. Sample DNA contamination with those cells causes decrease of LINE-1 hypomethylation level and may result in failure to detect the differences between early and advanced cancer.

Our method enabled methylation analysis of sample DNA from small areas about 2x10\textsuperscript{5} μm\textsuperscript{2} and this enabled to analyze heterogeneity of LINE-1 methylation status within the tumor. The comparison between the deepest part and the surface of the tumor revealed that tumor heterogeneity of LINE-1 methylation status is relatively subtle. The results suggest that colorectal tumor biopsy specimens collected during colonoscopy are representative can be used for a tumor's LINE-1 methylation status analysis. LINE-1 hypomethylation levels differed significantly between node positive and node negative cancers and the AUC was 0.69 to predict node positive cancer from whole samples according to the ROC curve analyses. The LINE-1 unmethylation index of colorectal tumor biopsy sample may therefore be useful to preoperatively predict lymph node status of colon cancer. Prospective studies are needed to confirm our study's findings.

In conclusion our study demonstrated that LINE-1 methylation status correlates with clinical stage of colon cancer and its accurate analysis with LCM or equivalent techniques is mandatory. The combination of LCM, on-cap SBM and AQAMA is a powerful method for such accurate analysis.
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References
