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의학박사 학위논문

Assessment of *LINE-1* and *Alu* hypomethylation level in colorectal cancer and precancerous lesion to disclose their clinicopathologic and prognostic implication

대장암과 그 전암성병변에서의 *LINE-1* 과 *Alu* 의 hypomethylation 분석 및 임상병리학적, 예후인자적 의미에 대한 연구

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이예영

A thesis of the Degree of Doctor of Philosophy

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Aug. 2015

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by
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A thesis submitted to the Department of Pathology
in partial fulfillment of the requirements for the
Degree of Doctor of Philosophy in Pathology at
Seoul National University College of Medicine

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Approved by Thesis Committee:

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ABSTRACT

Introduction: Recent studies have tried to understand the comprehensive tumorigenesis of Colorectal cancers (CRCs) which include the genetic and epigenetic pathways. Even though the epigenetic pathways are more actively studied than before, the researches related to the global DNA hypomethylation in CRCs has not been done much so far.

Genomic hypomethylation in carcinomas is related with the hypomethylation of repetitive DNA elements which comprise 45% of the human genome. In association with tumorigenesis, CpG sites in the repetitive DNA elements tend to undergo demethylation, leading to generalized diffuse genomic hypomethylation and consequentially genomic instability, activation of transposable element and oncogene. Recent studies have demonstrated that a higher degree of Long interspersed nuclear element-1 (*LINE-1*) hypomethylation is related to poor prognosis of CRCs.

In this study, I have analyzed *LINE-1* and *Alu* methylation level in Korean CRCs and precancerous lesions based on the results of previous studies. In particular, the relation between *LINE-1* and *Alu* methylation and clinical prognosis in MSI

positive CRC, and the relation between *LINE-1* hypomethylation and clinical prognosis in stage III or high-risk stage II colorectal cancers treated with adjuvant combination therapy of 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX) were elucidated.

Methods: Analysis was done in 767 CRCs, and of those 207 Microsatellite instability positive (MSI+) CRCs and 154 precancerous lesions for their methylation levels of *LINE-1* and *Alu* repetitive DNA elements using pyrosequencing assay. The correlation of the tumoral *LINE-1* and *Alu* methylation change with clinicopathological information including survival data was analyzed.

I also analyzed 427 resected stage III or high-risk stage II CRCs for their status in L1 methylation, CpG island methylator phenotype (CIMP), microsatellite instability (MSI), and *KRAS*/*BRAF* mutation. Four CpG sites were assayed for L1 methylation using pyrosequencing. The average methylation level of L1 methylation on combined two CpG sites (CpG sites 2 and 3) was obtained for each CRC case.

Results: The hypomethylation of *LINE-1* and *Alu* did not have the value as a prognostic factor when the analysis was done for the whole colorectal cancer samples. However in MSI+ CRCs, univariate survival analysis showed that low *Alu* methylation status (<18.60%) and low *LINE-1* methylation status (<53.00%) were significantly associated with shorter overall survival time (log-rank test, P = 0.009 and P < 0.001, respectively). Multivariate analysis using nine parameters (*Alu* methylation status, *LINE-1* methylation status, age, disease stage [tumor, node, metastasis staging system], differentiation, Crohn-like lymphoid reaction, *KRAS/BRAF* mutation status, CIMP status, and peritumoral lymphocytic infiltration), which were significantly prognostic in MSI+ CRCs, revealed that low *LINE-1* methylation status was an independent prognostic factor of MSI+ CRCs (P = 0.009), whereas low *Alu* methylation status was not.

L1 hypomethylation was closely associated with nodal metastasis but did not show any association with age of onset, gender, tumor subsite, tumor differentiation, mucinous histology, lymphatic emboli, venous invasion, perineural invasion, T stage, and *KRAS/BRAF* mutation in patients with stage III or high-

risk stage II CRCs treated with adjuvant FOLFOX. Multivariate analysis revealed that L1 hypomethylation as well as mucinous histology, T stage, N stage, lymphatic emboli, and KRAS mutation was an independent prognostic parameter heralding poor prognosis in stage III or high-risk stage II CRCs treated with adjuvant FOLFOX.

Conclusions: Clinical outcomes of MSI+ CRCs depend on *LINE-1* methylation status, suggesting that lower *LINE-1* methylation status serves as a significant prognostic parameter of adverse prognosis in MSI+ CRCs.

Tumoral L1 hypomethylation correlated independently with poor prognosis in patients with stage III or high-risk CRCs treated with adjuvant FOLFOX, and could be utilized as biomarker identifying a subset which might be resistant against adjuvant FOLFOX.

Keywords: Colorecral cancer, Adjuvant therapy, Long interspersed element-1 (*LINE-1*), *Alu*, DNA methylation, MSI, pyrosequencing, prognosis

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the United States, and the second most common in men, the third most common in women in Korea. Although the incidence rate is high, the relative death rate compared to the whole cancers is 10.8% and five year survival rate is gradually increased from 54.8% to 72.6% from 1993 to 2010.(1)

Early detection could be attributed to the cause of survival improvement. However, active research on the tumorigenesis, molecular subtype and prognostic factors of CRC and its clinical application are thought to be more critical to the survival improvement. Though targeted therapy using monoclonal antibody or small molecular inhibitor drug related to EGFR would be a representative example, molecular markers of colorectal cancer elucidated so far is not sufficient for realizing personalized medicine.(2) This could be attributes to the fact that the studies of the molecular pathology of colorectal cancer had been mostly concentrated on the chromosomal instability pathway and microsatellite instability pathway. Chromosomal instability–positive CRCs are characterized by alterations in the

number and structure of chromosomes and accumulations of genetic mutations in tumor-related genes. Microsatellite instability (MSI)-positive (MSI+; often referred to as MSI-high or MSI-H) CRCs feature frameshift mutations in genes containing small runs of nucleotide repeats in exonic coding regions.(3)

Recent studies on molecular pathogenesis of colorectal cancer have recognized epigenetic instability pathway which is due to the abnormal DNA methylation as one of the important tumorigenesis pathways together with chromosomal instability pathway and microsatellite instability pathway. New focusing on the epigenetic mechanism in tumorigenesis could contribute to the development of a useful molecular prognostic factor of CRCs.

DNA methylation plays an important role in several physiological processes including X chromosome inactivation, genomic imprinting, suppression of gene expression, regulation of cell differentiation and development, and maintenance of chromosome structure and integrity. However, almost all cancers have DNA methylation abnormality including CpG island (promoter CpG region) hypermethylation and global DNA

hypomethylation, causing repression of tumor suppressor gene(4-6) or genomic instability(7-10): CpG island methylator phenotype (CIMP)-positive (CIMP+; often referred to as CIMP-high or CIMP-H) CRCs refer to a subset of CRCs characterized by widespread concurrent hypermethylation of multiple promoter CpG island loci. Therefore, understanding of DNA methylation in cancers could be utilized in clinical diagnosis and treatment.(11)

Recent comprehensive studies interrogating the genetic and epigenetic changes of colorectal cancer indicate that the CIMP and chromosomal instability are inversely correlated in colorectal cancers,(12) that CIMP underlies sporadic microsatellite instability and is tightly associated with *BRAF* mutation,(13) and that mutations in both *KRAS* and *BRAF* may contribute to the CIMP.(14) These researches would contribute to the development of molecular prognostic factors of colorectal cancers. Even though the epigenetic pathways are actively studied as described above, the researches related to the global DNA hypomethylation CRCs has not been done much so far.

Genomic hypomethylation in carcinomas is related with the hypomethylation of transposable repetitive DNA elements

which comprise 45% of the human genome. Long interspersed nuclear element-1 (*LINE-1*) and *Alu* comprise about 28% of the human genome. *LINE-1* and *Alu* are retrotransposons spread in the human genome with 500, 000 copies of 5kb length, and 1,000,000 copies of 300bp length, respectively. CpG sites located within the repetitive DNA elements are heavily methylated in normal cells, which is important for suppression of transposon activity and thus maintenance of genomic stability.(15-20) However, in association with tumorigenesis, CpG sites in the repetitive DNA elements tend to undergo demethylation, leading to generalized diffuse genomic hypomethylation and consequentially genomic instability,(7, 10, 21) activation of transposable element(22, 23) and oncogene.(24-26) Methylation levels of *LINE-1* or *Alu* determined by PCR-based assays correlate well with 5-methylcytosine content in the human genome and thus can be used as a surrogate for genome-wide methylation content.(27) Recent studies have demonstrated that a higher degree of *LINE-1* hypomethylation is related to poor prognosis of CRCs or colon cancers.(28, 29) MSI+ CRCs show higher *LINE-1* methylation levels compared with MSI-negative CRCs.(30)

However, a wide variation of *LINE-1* methylation levels exists within MSI+ CRCs.(31)

Approximately 45% of CRC patients who have undergone curative surgery alone ultimately relapse and die of metastatic disease (32). Since randomized trials conducted in the 1980s reported that 5-fluorouracil (5-FU)-based adjuvant treatment could decrease the chance of death by approximately 30% (33), 5-FU-based adjuvant chemotherapy has been widely used for stage III (node-positive) colon cancers (34, 35). Based on the results of large adjuvant phase III trials supporting the survival benefit of adding oxaliplatin to 5-fluorouracil (5-FU), oxaliplatin in combination with 5-FU and leucovorin (FOLFOX) has become the worldwide standard of care for patients with stage III colon cancers (36, 37). However, despite the efficacy of FOLFOX, a significant proportion of stage III patients receive adjuvant chemotherapy without resultant benefit (38). The identification of biomarkers to predict tumor response to FOLFOX adjuvant therapy is important for the personalized treatment of CRC, thereby enhancing tumor-response and survival rates.

In this study, *LINE-1* and *Alu* methylation levels were analyzed in Korean colorectal cancers and precancerous lesions using pyrosequencing assay, which enabled determination of chronological changes of *LINE-1* and *Alu* in multistep colorectal carcinogenesis and disclosure of the relationship of *LINE-1* and *Alu* methylation level with clinicopathological features and molecular subtypes of colorectal cancers. In particular, the relation between *LINE-1* and *Alu* methylation and clinical prognosis in MSI positive colorectal cancer, and the relation between *LINE-1* hypomethylation and clinical prognosis in stage III or high-risk stage II colorectal cancers treated with adjuvant combination therapy of FOLFOX were elucidated.

MATERIALS AND METHODS

1. Tissue Samples

This study was approved by the Institutional Review Board of Seoul National University College of Medicine. From the surgical files in the Department of Pathology, Seoul National University Hospital, Seoul, Korea and the Department of Diagnostic Pathology, Asan Medical Center, Seoul, Korea, we retrieved formalin-fixed, paraffin-embedded archival tissue blocks of CRCs from patients who underwent curative surgery from 2004 to 2006, and of polyps from patients who underwent polypectomy in 2007. The total number of colectomy cases was 989 (Seoul National University Hospital) and 2478 (Asan Medical Center); of these, 78 (7.9 %) and 170 (6.9 %) cases, respectively, were found to be MSI+. Of the 989 resected cases from Seoul National University Hospital, 767 cases were available for the molecular study and of those 248 MSI+ cases, 207 were available for the molecular study. The total number of polypectomy cases was 154 (Seoul National University Hospital). Electronic medical records were reviewed for clinicopathological information including staging according to the tumor, node, metastasis staging system (TNM), and

adjuvant therapy. Staging of the disease was classified according to the sixth edition of guidelines of the American Joint Committee on Cancer.

I also used formalin-fixed paraffin-embedded archival tissue blocks from stage III or high-risk stage II CRC patients who underwent curative resection between April 2005 and December 2011 and received at least 6 cycles of adjuvant FOLFOX chemotherapy at Seoul National University Hospital. The chemotherapy regimen was FOLFOX-4 or modified FOLFOX-6, and the therapy was planned for a total of 12 cycles. Patients were assessed every 2 weeks during the treatment and then at least every 6 months for 5 years. The recurrence was diagnosed using imaging and, if necessary, via biopsy. This cohort of 521 patients has been reported in previous studies (39, 40), and as previously described, the inclusion criteria in addition to tumor stage were age over 18 years and adenocarcinoma histology. Patients were defined to have high-risk stage II CRC if they had any of the following: T4 lesion, obstruction or perforation, lymphovascular invasion, perineural invasion, or poorly differentiated histology. The exclusion criteria were as follows: previous chemotherapy for

CRC, previous radiotherapy for CRC, signet ring cell histology, distant metastasis, and history of any other malignancies within 5 years. Out of the eligible 521 cases, 427 were available for methylation analysis of Long interspersed element-1 (*LINE-1*, L1). Electronic medical records were reviewed for clinicopathological information.

2. Histologic examination

Through microscopic examination of representative sections of tumors, two pathologists evaluated the following parameters without knowledge of *Alu* and *LINE-1* methylation status: histological differentiation (World Health Organization), Crohn-like lymphoid reaction, and peritumoral lymphocytic reaction.

3. Immunohistochemistry

Tumor-infiltrating lymphocytes were counted by immunostaining with anti-CD8 (mouse, DAKO). Under a light microscope (400×), five visual fields were randomly selected, and the number of positively stained cells were counted. The mean number of tumor-infiltrating lymphocytes per high-

power field for each tumor was then calculated by dividing the total number of tumor-infiltrating lymphocytes by five.

4. DNA Extraction and Determination of MSI

After microscopic examination of the tissue glass slides from MSI+ CRCs, I marked tumor areas that represented the main histology of the individual cases and were the richest in tumor cells and dissected them manually with knife blades. The dissected tissues were collected into microtubes containing lysis buffer and 0.4 μg/μl proteinase K, and these tubes were incubated at 55 °C for 24-48 h. Because MSI testing is incorporated into routine clinical practice in both Seoul National University Hospital and Asan Medical Center, the MSI status of these tumors had been analyzed previously before the present study and determined by the NCI reference markers: BAT25, BAT26, D2S123, D5S346, and D17S250. Samples were classified as MSI+ when at least two loci showed MSI.

5. KRAS and BRAF Mutation Analysis

KRAS codons 12 and 13 and BRAF codon 600 were assessed by PCR and sequence analysis as described.(42, 43)

6. Bisulfite Modification and CIMP Analysis

Using a DNA methylation kit (Zymo Research, Orange, CA, USA), I performed bisulfite modification of the lysed tissue solution according to the protocol. I used the eight-marker panel (CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1) and determined the CIMP status in 207 MSI+ CRCs using the MethyLight assay. DNA methylation at each marker was calculated as a percent of methylated reference (PMR): PMR $=100\times[(\text{methylated reaction}/Alu)_{\text{sample}}/(\text{methylated reaction}/Alu)_{M.SssI\text{-reference}}]$. I considered a CpG island locus to be methylated when an exponential amplification curve was generated and the PMR v*alu*e was >4. CIMP tumors were categorized according to their methylation status, as follows: tumors with methylation at ≥5 markers, CIMP-H; tumors with methylation at 1-4 markers, CIMP-L; no loci, CIMP-0.

7. Analysis of Methylation Status in Repetitive DNA Elements

LINE-1 and *Alu* methylation was quantified using bisulfite-PCR and pyrosequencing. In brief, bisulfite-modified DNA

samples were PCR-amplified with oligonucleotide primers designed toward a consensus *LINE-1* or *Alu* sequence, which allowed amplification of a representative pool of repetitive elements to serve as a surrogate for global DNA methylation changes. I used the same oligonucleotide primers designed by the Issa group for pyrosequencing.(44) The primers and PCR conditions are listed in Table 1. The PCR product was purified and quantified using the PyroMark Q24 System (Biotage AB, Uppsala, Sweden). The degree of methylation was expressed as the percentage of 5′ -methylated cytosines over the sum of methylated and unmethylated cytosines. The ratio of C to the sum of C and T nucleotides was evaluated for *LINE-1* methylation, and the ratio of G to the sum of G and A nucleotides was evaluated for *Alu* methylation. (Fig.1) DNA from SNU-16, SNU-484, and SNU-601 cell lines as well as SssI-treated DNA were used as controls for optimizing the assay. Whole-genome amplified DNA was used as a negative control. The experiments were repeated twice, and mean values were taken from these separate experiments. Methylation levels at three consecutive CpG sites were evaluated.

8. Statistical analysis

Statistical analyses were performed using the statistics program SPSS software (SPSS version 15.0, Chicago, IL, USA). Because data of *Alu* or *LINE-1* methylation levels did not follow the normal distribution, mean values between two groups or among three or more groups were compared using Mann-Whitney U test and Kruskal-Wallis test, respectively. Pearson correlation was used to evaluate the correlations between ages and *LINE-1* or *Alu* methylation levels and between *LINE-1* and *Alu* methylation levels. For the study about MSI+ CRCs, survival was measured from the data of resection of CRC to the date of death or the last clinical follow-up time (before 31 May 2010). The average follow-up time (from surgery to death or the last follow-up time) was 46 months (range 1-76 months). Overall survival rates were assessed with the Kaplan-Meier log-rank test. All of the measured values of *Alu* or *LINE-1* methylation level were tested as a cutoff value to divide the methylation-high group and methylation-low group which were subjected to Kaplan-Meier log-rank test. Through these serial analyses, I obtained the optimal cutoff values of *Alu* or *LINE-1* methylation level which produced the lowest P-value. To

control confounding factors, I used Cox proportional hazards models to calculate hazard ratio of death according to *LINE-1* or *Alu* methylation status of tumor, adjusting for age at diagnosis, tumor location, gross type, tumor grade, Crohn-like lymphoid reaction, peritumoral lymphocytic reaction, TNM stage, CIMP, and *KRAS/BRAF* mutation status. These covariates except for peritumoral lymphocytic reaction were found to be statistically significant prognostic parameters in Kaplan-Meier log-rank test. Peritumoral lymphocytic reaction was a marginally significant prognostic parameter in Kaplan-Meier log-rank test. To avoid overfitting, variables in the final model were selected using backward stepwise elimination with a threshold of $P = 0.05$.

For the study about stage III or high-risk CRCs treated with adjuvant FOLFOX, clinical data were last updated in January 2014. Disease free survival (DFS) was calculated from the date of surgery to the date of recurrence or death by Kaplan-Meier analysis using quartile groups of L1 methylation levels, and the significance of differences among the four groups was determined using the log-rank test. I calculated the hazard ratio of death corresponding to L1 methylation using Cox

proportional hazards models. All tests were two-sided, and statistical significance was set at P <0.05.

Table 1. Primer sequences and PCR conditions used for pyrosequencing.

	Primer		Tm (℃)
L1	Forward	5' -TTTTGAGTTAGGTGTGGGATATA-3'	52
	Reverse	5' -biotin- AAAATCAAAAAATTCCCTTTC-3'	
	Sequencing	5' -AGTTAGGTGTGGGATATAGT-3'	
ALU	Forward	5' -biotin- TTAAAAATATAAAAATTAGT-3'	54, 52, 50, 48
	Reverse	5' -CCAAACTAAAATACAATAA-3'	
	Sequencing	5' -AATAACTAAAATTACAAAC-3'	

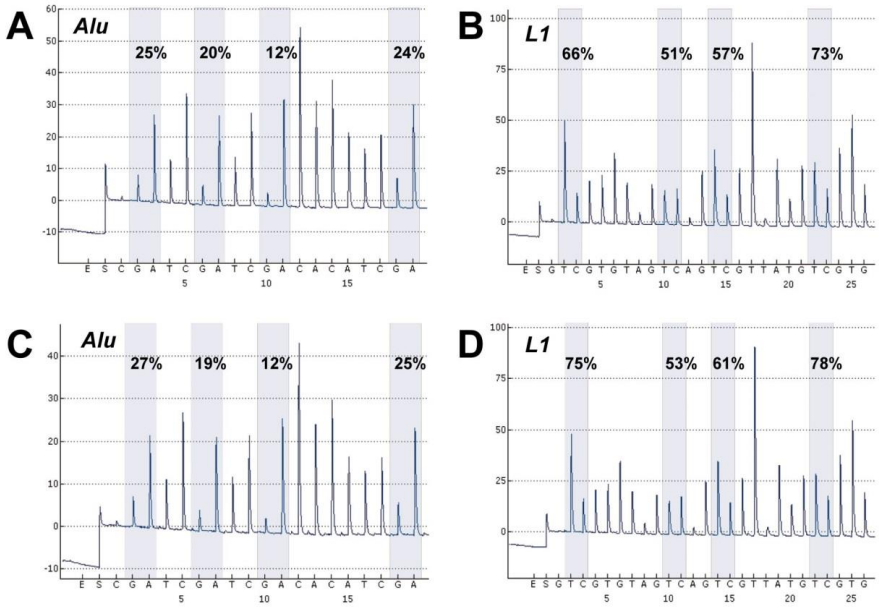

Figure 1. Pyrogram of *LINE-1* and *Alu* methylation.

The degree of methylation was expressed as percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Methylation levels at three consecutive CpG sites (CpG sites 1 – 3) were evaluated. *Alu* and *LINE-1* methylation statuses of a cancer tissue sample (A and B). *Alu* and *LINE-1* methylation statuses of a normal tissue sample (C and D)

RESULTS

LINE-1 and *Alu* methylation level in colorectal cancer and precancerous lesion.

The methylation level of *LINE-1* and *Alu* in cancer tissue was significantly lower than the normal mucosa; however, there was a large difference among tumor samples. Although genomic DNA methylation content is known to be decreased in precancerous lesions, little is known regarding the methylation status of *LINE-1* and *Alu* in precancerous lesions. Methylation levels of *LINE-1* and *Alu* were analyzed in 154 polypectomy cases using pyrosequencing. A decrease in the methylation level of *LINE-1* and *Alu* was found to occur at the stage of adenoma. Tumoral *LINE-1* hypomethylation level was observed at the low grade dysplasia step, while tumoral *Alu* hypomethylation was observed at the high grade dysplasia. *LINE-1* methylation level was significantly lower in villous adenomas and tubulovillous adenomas than in tubular adenomas with low grade or high grade dysplasia. *Alu* methylation level was lower in villous adenoma than in tubulovillous adenomas and tubular adenomas with high grade dysplasia. (Fig. 2)

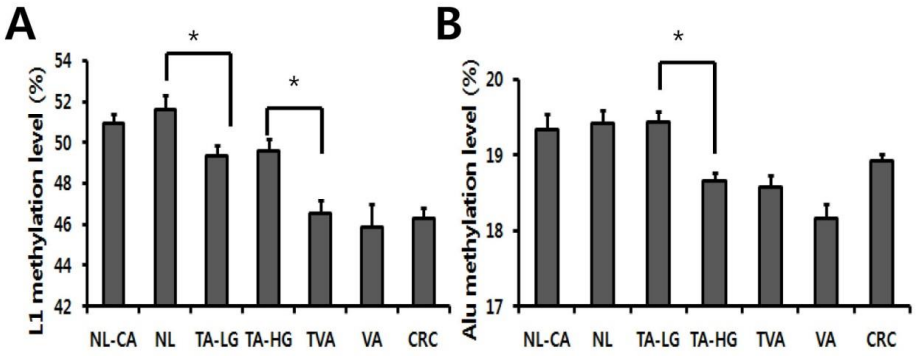

Figure 2. Methylation levels of *LINE-1* (A) and *Alu* methylation levels (B) in multistep colorectal carcinogenesis. NL-CA, normal colorectal mucosa from cancer patients; NL, normal colorectal mucosa from non-cancer subjects; TA-LG, tubular adenoma, low grade; TA-HG, tubular adenoma, high grade; TVA, tubulovillous adenoma; VA, villous adenoma; CRC, colorectal cancer. Asterisk indicates statistically significant difference between means of two groups ($P < 0.001$)

Correlation of *Alu* and *LINE-1* Methylation Status with clinicopathological characteristics in the 767 CRC cases

In cancer samples, *LINE-1* and *Alu* methylation levels were decreased compared to the respective one of normal mucosa.

To evaluate whether tumoral *LINE-1* or *Alu* hypomethylation may have prognostic significance, 767 cases of CRC were analyzed for their methylation status in *LINE-1* and *Alu* using pyrosequencing. The number of cases available for the analysis of methylation status of *LINE-1* and *Alu* was 461 and 416, respectively. *Alu* methylation levels ranged from 16.21 to 21.65 % (mean 19.34 %), and *LINE-1* methylation levels ranged from 5.26 to 69.59 % (mean 49.01 %). Rectal tumors showed lower *LINE-1* methylation levels (mean 47.25%) compared with right-colon (mean 50.58%) tumors or left-colon tumors (mean 49.34%, p=0.004). Poorly differentiated tumors exhibited lower *Alu* methylation levels compared to well-differentiated or moderately differentiated tumors, but the difference was statistically insignificant (P=0.190).

Relationships between tumoral *LINE-1* or *Alu* hypomethylation and patient survival

All possible cutoff values of *Alu* or *LINE-1* methylation level to divide the methylation-high group and methylation-low group were tested to determine the optimal cutoff values that produced the greatest prognostic value in a univariate survival

analysis. Tumoral *LINE-1* or *Alu* hypomethylation did not have prognostic value when the analysis was done for the whole colorectal cancer samples. (Fig 3, Table 3)

However, gross type, TNM stage, differentiation, Crohn-like lymphoid reaction, peritumoral lymphocytic infiltration, tumor-infiltrating lymphocytes, CIMP status were significant prognostic parameters in the univariate survival analysis. (Table 3)

Methylation levels of *LINE-1* varied depending on molecular subtypes of colorectal cancers. The whole colorectal cancers were classified to four groups according to the CIMP status and MSI status such as CIMP-/MSI-, CIMP-/MSI+, CIMP+/MSI-, and CIMP+/MSI+. *LINE-1* methylation level showed different dispersion depending on the molecular subtypes such that CIMP+/MSI+ subtype showed higher methylation level than CIMP-/MSI- subtype. ($P < 0.001$)(Fig 4) Because molecular subtypes of colorectal cancers showed differences in terms of patient survival as well as methylation levels of *LINE-1*, survival analysis had to be performed for each molecular subtype of colorectal cancers. Although we could not find any prognostic significance in molecular subtype of MSI-, we found

that for MSI+ CRCs, tumoral *LINE-1* hypomethylation correlated with lower overall survival rate.

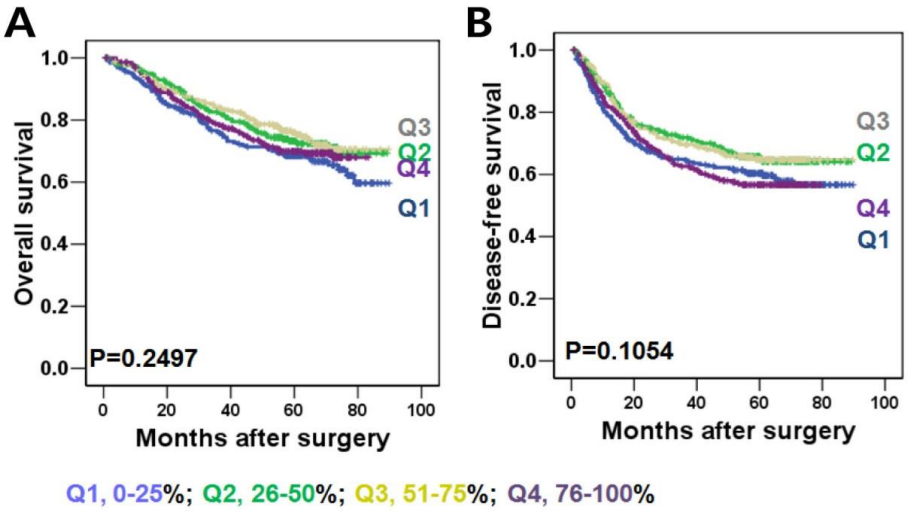

Fig 3. Kaplan Meier survival analysis in colorectal cancer patients according to *LINE-1* or *Alu* methylation levels. Overall survival analysis (A) and disease-free survival (B).

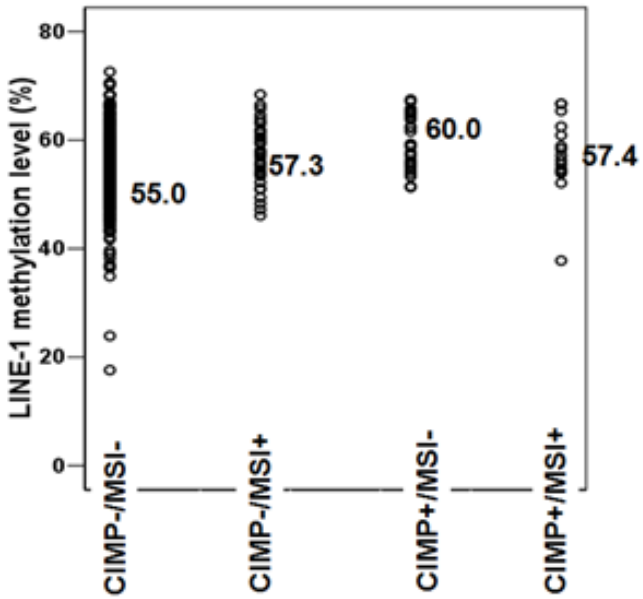

Fig 4. *LINE-1* methylation levels in four molecular subtypes of colorectal cancers generated by both CIMP and MSI statuses. *LINE-1* methylation level is highest in CIMP+/MSI- subtype, whereas *LINE-1* methylation level was lowest in CIMP-/MSI- subtype (ANOVA test, $P < 0.001$). MSI+ subtypes showed *LINE-1* methylation levels in between two molecular subtypes (CIMP+/MSI- and CIMP-/MSI-). However, MSI+ subtype showed similar *LINE-1* methylation level regardless of CIMP status.

Correlation of *Alu* and *LINE-1* Methylation Status with Clinicopathological Parameters in MSI+ CRCs.

Alu and *LINE-1* methylation levels were measured in 207 MSI + tumors. *Alu* methylation levels ranged from 14.13 to 22.52 % (mean 19.75 %), and *LINE-1* methylation levels ranged from 28.79 to 70.67 % (mean 57.68 %). There was a positive correlation between *Alu* and *LINE-1* methylation levels (Pearson correlation coefficient, $r^2 = 0.225$, P = 0.001; Fig. 5). No correlation was found between patient age and *Alu* methylation level or *LINE-1* methylation level of MSI + tumors ($r^2 = 0.094$ and $r^2 = 0.034$, respectively; both P> 0.05). Gross type was significantly associated with *Alu* or *LINE-1* methylation level, and ulceroinfiltrative tumors showed lower *Alu* or *LINE-1* methylation levels than the respective methylation levels in polypoid or ulcerofungating tumors (P< 0.05; Table 4). Left-colon tumors showed lower *LINE-1* methylation levels compared with right-colon tumors or rectal tumors (P = 0.028). Poorly differentiated tumors exhibited lower *Alu* methylation levels compared with well-differentiated or moderately differentiated tumors, but the difference was statistically insignificant (P = 0.209). Although the relationship

of *Alu* or *LINE-1* methylation levels with TNM stage was not statistically significant, there was a tendency of decreasing *Alu* or *LINE-1* methylation levels with increasing tumor stage.

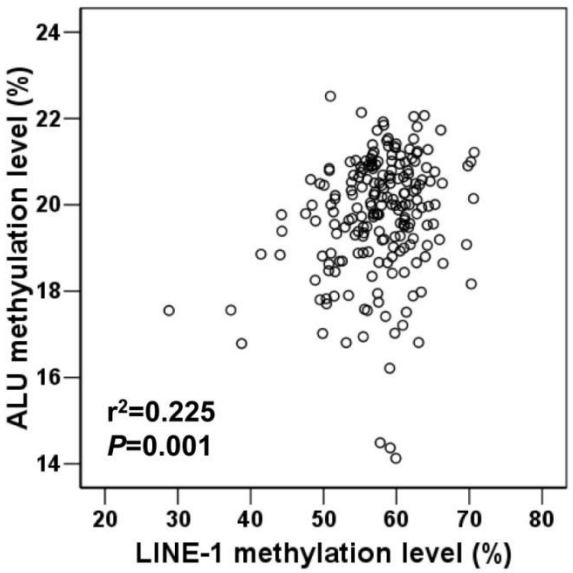

Figure 5. There was a positive correlation between *Alu* and *LINE-1* methylation levels (Pearson correlation coefficient, r^2 = 0.225, P = 0.001)

Correlation of *Alu* and *LINE-1* Methylation Levels with Molecular Features of MSI+ CRCs

To assess the correlation between CIMP status and *LINE-1* or *Alu* methylation levels, we analyzed MSI+ CRC cases (n = 207) for their CIMP status using the MethyLight assay and then classified 62 (30.0%), 106 (51.2%), and 39 (18.8 %) as CIMP-H, -L, and -0, respectively. MSI+ CRCs showed different *LINE-1* methylation levels depending on CIMP status: 59.57, 57.13, and 56.18 % for CIMP-H, -L, and -0, respectively (P = 0.001). However, no difference was noted in the *Alu* methylation levels among these three subtypes. Because *KRAS* mutation is mutually exclusive with *BRAF* mutation, MSI+ CRCs could be grouped into three subgroups: (1) *KRAS* mutation-positive subgroup (18.2 %); (2) *BRAF* mutation-positive subgroup (9.1 %); (3) wild-type *KRAS*/*BRAF* subgroup (72.7 %). The *LINE-1* methylation level was highest in the *BRAF* mutation subgroup and lowest in the wild-type *KRAS*/*BRAF* subgroup (P = 0.049). However, *Alu* methylation levels were similar in the three subgroups. Because *BRAF* mutation was found only in the CIMP-H subgroup, it was unclear whether the high *LINE-1* methylation level was related

to CIMP-H or to *BRAF* mutation. When we compared *LINE-1* methylation levels within CIMP-H tumors with respect to *KRAS*/*BRAF* mutation status, no difference was found in the CIMP-H subgroup with respect to the combined *KRAS*/*BRAF* mutation status. However, for the CIMP-L subgroup, *KRAS* mutation-positive CIMP-L tumors tended to show higher *LINE-1* methylation levels than those of *KRAS* mutation-negative CIMP-L tumors (P = 0.057).

Alu and *LINE-1* Methylation Status versus Patient Survival in MSI+ CRCs.

All possible cutoff values of *Alu* or *LINE-1* methylation level to divide the methylation-high group and methylation-low group were tested to determine the optimal cutoff values that produced the greatest prognostic value in a univariate survival analysis. The optimal cutoff values were 18.60 and 53.00 % for *Alu* and *LINE-1*, respectively. Low *LINE-1* methylation status (*LINE-1*-low, <53.00 %) was closely associated with shortened overall survival time (P < 0.001; Fig. 6a. Low *Alu* methylation status (*Alu*-low, <18.60 %) was also closely associated with shortened overall survival time (P = 0.009; Fig. 6b). Besides *Alu* methylation status and *LINE-1* methylation

status, patient age, TNM stage, differentiation, Crohn-like lymphoid reaction, *KRAS/BRAF* mutation status, and CIMP status were all significant prognostic parameters in the univariate survival analysis. Peritumoral lymphocytic infiltration was marginally significant prognostic parameter (P = 0.078). These nine parameters were entered into the multivariate analysis to determine whether *Alu*-low or *LINE-1*-low was an independent prognostic factor for MSI+ CRC. *LINE-1*-low appeared as an independent factor for poor prognosis (P = 0.009), whereas *Alu*-low did not (Table 5). A stage-specific survival analysis showed that the stage III CRCs with *LINE-1*-low had worse clinical outcomes than stage III CRCs with high *LINE-1* methylation status (*LINE-1*-high, ≥53.00 %) (P = 0.0133; Fig. 7). Stage II CRCs with *LINE-1*-low tended to correspond with poorer clinical outcomes than that of corresponding stage II CRCs with *LINE-1*-high.

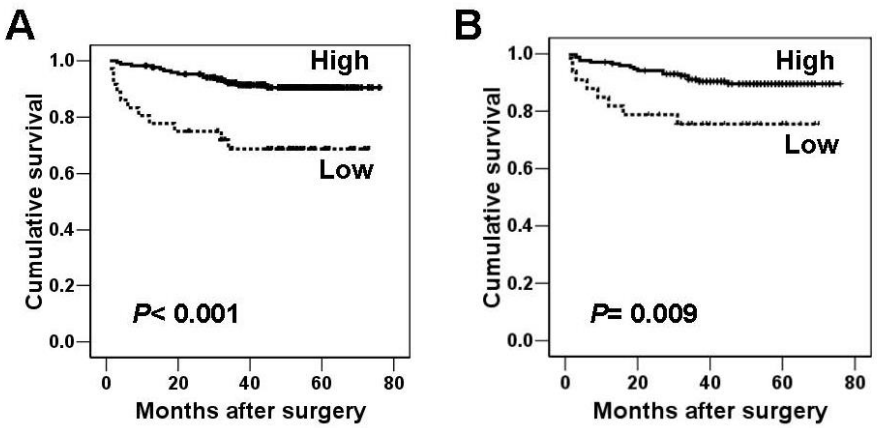

Fig 6. Kaplan-Meier survival analysis with log-rank test. Survival analysis was performed for patients with *LINE-1*-low (*LINE-1* methylation level <53.00 %, n = 36) and *LINE-1*-high (*LINE-1* methylation level ≥53.00 %, n = 171) CRCs (A). Survival analysis for patients with *Alu*-low (*Alu* methylation level <18.60 %, n = 51) and *Alu*-high (*Alu* methylation level ≥18.60 %, n = 154) CRCs (B).

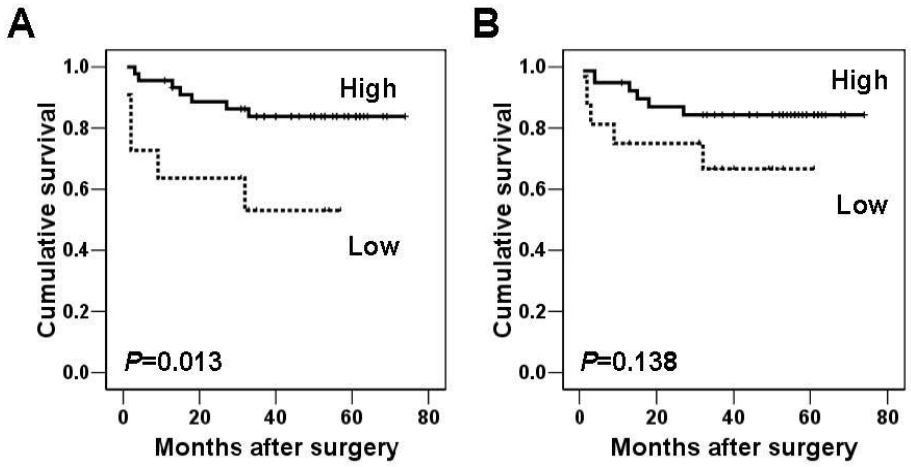

Fig 7. Kaplan-Meier survival analysis (log-rank test) in stage III tumors. Lower *LINE-1* methylation status was closely associated with shorter survival time (P = 0.013) (A). Lower *Alu* methylation status tended to show an association with shorter survival time, but the association was not statistically significant (P = 0.138) (B).

Table 2. Methylation levels of repetitive DNA elements (*Alu* and *LINE-1*) according to clinicopathological parameters in 767 CRCs

Characteristic		No. of cases	Alu Mean	Alu SD	Alu P-value	No. of cases	L1 Mean	L1 SD	L1 P-value
Gender									
M		254	19.33	0.84	0.811	273	48.88	9.59	0.588
F		162	19.36	1.01		187	49.12	10.1	
Age									
≤55 years		123	19.28	0.96	0.170	145	48.96	9.64	0.946
≥56 years		293	19.37	0.88		316	49.04	9.93	
Gross type									
Polypoid		37	19.61	0.79	0.155	46	50.1	9.82	0.027
Ulcerofungating		91	19.39	0.93		97	46.68	11.2	
Ulceroinfiltrative		279	19.28	0.91		309	49.76	9.28	
Site									
Right colon		117	19.3	0.92	0.404	128	50.58	10.2	0.004
Left colon		170	19.33	0.87		186	49.34	9.67	
Rectum		129	19.39	0.95		147	47.25	10.6	
Differentiation									
Well		27	19.24	1.08	0.190	31	46.43	12.2	0.687
Moderate		373	19.37	0.9		411	49.24	9.55	
Poor		16	18.99	0.82		19	48.38	11.6	
T category									
	1	12	19.5	0.83	0.058	14	55.21	7.19	0.094
	2	62	19.45	0.69		68	49.69	9.45	
	3	308	19.36	0.95		340	48.68	9.68	
	4	34	18.96	0.85		39	48.47	12	
N category									
	0	146	19.87	1.36	0.038	206	50.01	0.85	0.097
	1	37	19.69	1.48		120	46.87	0.95	
	2	24	19.11	1.42		90	49.26	0.97	
M category									
	0	345	19.33	0.9	0.736	386	48.98	9.66	0.427
	1	71	19.39	0.93		75	49.22	10.8	

TNM staging								
	Ⅰ	62	19.45	0.73	0.172	69	51.35	9.39	0.020
	Ⅱ	128	19.42	0.91		146	49.34	9.97	
	Ⅲ	156	19.22	0.95		169	47.72	9.32	
	Ⅳ	70	19.38	0.93		74	49.1	10.8	

Table 3. Univariate and multivariate prognostic analyses of 767 CRCs

Characteristic	No. of cases[c]	Univariate analysis Mean survival (mo) (95 % CI)	P-value [a]	Multivariate analysis HR (95 % CI)	P-value [b]
Age					
≤55 y	239	73 (69-77)	0.250		
≥56 y	528	67 (65-70)			
Location					
Right colon	211	65 (61-70)	0.167		
Left colon	328	70 (67-74)			
Rectum	228	71 (67-75)			
Gross type					0.019
Polypoid	84	73 (68-78)	< 0.001		
Ulcerofungating	418	72 (70–75)			
Ulceroinfiltrative	260	62 (58-66)		1.318 (1.047-1.660)	
Differentiation					0.001
Differentiated	737	70 (68-73)	< 0.001		
Poorly differentiated	30	39 (26-53)		2.332 (1.406-3.868)	
Crohn-like lymphoid reaction					0.012
Absent	371	66 (63-69)	0.004	0.695 (0.523-0.925)	
Present	396	72 (69-75)			
Peritumoral lymphocytic reaction					0.956
Absent or mild	621	68 (66-71)	0.040	0.989 (0..669-1.463)	
Marked	146	72 (68-77)			

Tumor-infiltrating lymphocytes					0.204
<7/HPF	529	67 (64-70)	0.007	0.810 (0.585-1.121)	
≥7/HPF	238	74 (70-77)			
TNM stage					<0.001
Ⅰ, Ⅱ	363	81 (79-83)	<0.001		
Ⅲ	270	70 (66-74)			
Ⅳ	134	37 (32-42)		3.105 (2.569-3.754)	
Postoperative chemotherapy					
No	259	67 (63-72)	0.453		
Yes	508	70 (67-73)			
CIMP					0.099
CIMP-0, -L	733	70 (68-72)	0.005	1.486 (0.928-2.377)	
CIMP-H	34	57 (47-67)			
KRAS/BRAF mutation[c]					
KRAS +/BRAF−	189	67 (63-72)			
KRAS −/BRAF+	44	66 (56-76)	0.578		
KRAS −/BRAF−	489	70 (67-73)			
Alu					
High (≥20 %)	96	74 (68-80)	0.133		
Low (<20 %)	320	67 (64-71)			
L1					
High (≥58 %)	67	69 (67-73)	0.167		
Low (<58 %)	394	60 (53-68)			

[a]Log−rank test
[b]Cox proportional hazard regression model
[c]Number of cases available for the analysis of methylation status of *LINE−1* and *Alu* was 461 and 416 respectively.

Table 4. Methylation levels of repetitive DNA elements (*Alu* and *LINE−1*) according to clinicopathological parameters in MSI + CRCs.

Characteristic	No. of	Alu			L1		
	cases	Mean	SD	P-value	Mean	SD	P-value
Gender							
M	126	19.69	1.36	0.269	58.15	5.08	0.561
F	81	19.85	1.46		56.96	6.79	
Age							
<56 years	100	19.59	1.39	0.077	57.57	5.52	0.709
≥56 years	107	19.91	1.39		57.78	6.1	
Gross type							
Polypoid	15	20.5	0.57	0.047	57.22	3.45	0.013
Ulcerofungating	171	19.74	1.41		58.25	5.2	
Ulceroinfiltrative	21	19.32	1.56		53.28	9.38	
Site							
Right colon	144	19.77	1.41	0.313	57.7	5.52	0.028
Left colon	34	19.41	1.49		55.72	7	
Rectum	29	20.04	1.18		59.83	5.2	
Differentiation							
Well	16	20.41	1.04	0.209	58.23	5.23	0.818
Moderate	137	19.78	1.32		57.9	5.2	
Poor	54	19.49	1.63		56.95	7.33	
T category							
1	10	20.47	0.93	0.135	57.25	3.42	0.252
2	23	20.18	0.95		59.63	4.5	
3	156	19.7	1.35		57.4	5.71	
4	18	19.25	2.16		57.83	8.62	
N category							
0	146	19.87	1.36	0.038	57.88	5.01	0.071
1	37	19.69	1.48		59.22	5.19	
2	24	19.11	1.42		54.08	9.23	
M category							
0	199	19.77	1.38	0.488	57.83	5.64	0.345
1	8	19.26	1.81		54.1	8.99	
TNM staging							
I	28	20.3	0.89	0.120	58.25	3.95	0.812
II	115	19.76	1.45		57.83	5.11	
III	56	19.53	1.4		57.59	7.24	
IV	8	19.26	1.81		54.1	8.99	

Table 5. Univariate and multivariate prognostic analyses of MSI+ CRCs

Characteristic		Univariate analysis		Multivariate analysis	
	No. of cases[c]	Mean survival (mo) (95 % CI)	P-value[a]	HR (95 % CI)	P-value[b]
Age					
≤55 y	100	72 (69–75)	0.018		
≥56 y	107	63 (58–67)			
Location					
Right colon	144	67 (64–71)	0.298		
Left colon	34	61 (55–68)			
Rectum	29	68 (64–71)			
Gross type					
Polypoid	15	No death	0.021		
Ulcerofungating	171	69 (66–72)			
Ulceroinfiltrative	21	48 (39–56)			
Differentiation					
Differentiated	153		<0.001	–	0.043
Poorly differentiated	54	58 (50–66)		2.535(1.031–6.236)	
Crohn-like lymphoid reaction					
Absent	70	62 (56–67)	0.024	2.281 (0.915–5.688)	0.077
Mild or marked	137	71 (68–74)		–	
Peritumoral lymphocytic reaction					
Absent or mild	160	66 (62–69)	0.075		
Marked	47	74 (70–77)			
Tumor-infiltrating lymphocytes					
<7/HPF	58	60 (54–65)	0.300		
≥7/HPF	149	69 (66–72)			
TNM stage					0.007
I, II	143	73 (70–75)	<0.001	–	
Ⅲ	56	61 (54–67)		3.486 (1.354–8.974)	0.010
Ⅳ	8	35 (14–56)		6.362 (1.691–	0.006

				23.931)	
Postoperative chemotherapy					
No	117	65 (61–69)	0.403		
Yes	90	70 (66–74)			
CIMP					
CIMP-0, -L	145	72 (70–74)	<0.001	–	0.037
CIMP-H	62	57 (51–64)		3.052 (1.067–8.727)	
KRAS/BRAF mutation[c]					0.096
KRAS +/BRAF−	36	70(68–72)	0.002	0.346 (0.045–2.667)	0.308
KRAS −/BRAF+	18	47(34–60)		2.940 (0.883–9.783)	0.079
KRAS −/BRAF−	145	68(65–72)		–	
Alu					
High (≥18.60 %)	172	70 (68–73)	0.009		0.093
Low (<18.60 %)	33	55 (46–64)		2.551 (0.858–7.343)	
L1					
High (≥53.00 %)	171	71 (69–73)	<0.001	–	<0.001
Low (<53.00 %)	36	54 (44–63)		6.634 (2.528–17.410)	

[a]Log−rank test
[b]Cox proportional hazard regression model
[c]In eight cases, mutation status in both *KRAS* and *BRAF* could not be determined.

Pyrosequencing assay for L1 methylation status in stage III or high-risk stage II CRCs treated with adjuvant FOLFOX.

For a total of 427 cases of CRC, methylation levels of L1 CpG sites 2 and 3 were distributed with a mean value of 52.8%, median value of 51.7%, SD of 8.46%, range of 29.8% to 78.7%, and interquartile range of 47.3% to 57.3%. Methylation levels of L1 CpG site 3 were distributed with mean value of 55.0%, median value of 54.2%, SD of 9.70%, range of 23.7% to 86.1%, and interquartile range of 48.6% to 60.3%. CRCs were grouped into quartile groups (Q1, Q2, Q3, and Q4) according to the methylation levels of L1. Methylation levels of L1 CpG sites 2 and 3 were divided into quartiles [Q1 (29.8%-47.2%, n=106), Q2 (47.3%-51.6%, n=107), Q3 (51.7%-57.2%, n=108), and Q4 (57.3%-78.7%, n=106)], and methylation levels of L1 CpG site 3 were divided into quartiles [Q1 (23.7%-48.6%, n=105), Q2 (48.6%-54.1%, n=108), Q3 (54.2%-60.3,% n=107), and Q4 (60.3%-86.1%, n=107)].

Relationships between the L1 methylation level and clinicopathological features in stage III or high-risk stage II CRCs treated with adjuvant FOLFOX.

When I analyzed associations between the methylation level of L1 and clinicopathological parameters, the average methylation level of CpG sites 2 and 3 or methylation level of CpG site 3 was closely associated with N stage and molecular subtype but not associated with age of onset, gender, tumor subsite, tumor differentiation, mucinous histology, lymphatic emboli, venous invasion, perineural invasion, T stage, and *KRAS/BRAF* mutation (Table 6). Low methylation status of L1 was closely associated with nodal metastasis, but the methylation level of L1 was comparable between N1 and N2 stage. The methylation level of L1 was significantly higher in the CIMP-H MSS subtype than in the other three molecular subtypes.

Survival depending on the methylation level of *LINE-1* in stage III or high-risk stage II CRCs treated with adjuvant FOLFOX.

After a median follow-up of 49.3 months, the 3-year and 5-year DFS of the entire cohort were 89.4% and 84.9%, respectively. In the Kaplan-Meier survival analysis with log rank test, quartile groups of L1 CpG site 3 did not show statistically significant difference in DFS (Fig 8). In contrast, quartile groups of L1 CpG sites 2 and 3 exhibited a significant

difference in DFS: Q1 group showed the lowest DFS, whereas Q4 group displayed the highest DFS (Fig.9). In a univariate analysis, compared to Q4 cases, Q1 cases experienced a significantly lower DFS rate (P = 0.018, HR 2.998, 95% CI 1.208-7.439). The Q2 and Q3 cases experienced a slightly, but not significantly, lower DFS rate. For the subsequent multivariate analysis, Q2, Q3, and Q4 were combined and defined as the hypermethylated group, while Q1 was defined as the hypomethylated group. A Kaplan-Meir log rank test revealed that in addition to low methylation status of L1 CpG sites 2 and 3, high T stage, high N stage, lymphatic emboli, venous invasion, perineural invasion, mucinous histology, and KRAS mutation were associated with shorter DFS time (Table 7). These parameters and low methylation status of L1 CpG sites 2 and 3 were incorporated into a multivariate Cox model, which revealed that L1 hypomethylation correlated independently with shorter DFS time (HR 2.000, 95% CI 1.115-3.585, p = 0.020) (Table 8).

Table 6. Relationships between the L1 methylation level and clinicopathological parameters of colorectal cancer patients.

Characteristic	Variable	No. of cases	methylation level (CpG 2 and 3), %	P value	methylation level (CpG site 3), %	P value
Age	<60 year	209	53.1	0.454	55.2	0.552
	≥60 year	218	52.5		54.7	
Sex	Male	263	52.5	0.330	54.6	0.320
	Female	164	53.3		55.6	
Tumor subsite	Right colon	139	52.3	0.620	54.5	0.623
	Left colon	261	53.1		55.3	
	Rectum	27	52.2		53.9	
Differentiation	Low	393	52.6	0.113	54.7	0.093
	High	34	55.0		57.6	
Venous invasion	Absent	385	52.7	0.636	54.9	0.752
	Present	42	53.4		55.4	
Lymphatic emboli	Absent	247	52.4	0.271	54.4	0.182
	Present	180	53.3		55.7	
Perineural invasion	Absent	331	52.5	0.141	54.7	0.371
	Present	96	53.9		55.7	
T stage	T1	9	52.8	0.379	56.3	0.460
	T2	34	50.4		52.4	
	T3	323	53.0		55.2	
	T4	61	52.7		55.0	
N stage	N0	63	55.1	0.048	58.0	0.021
	N1	252	52.1		54.2	
	N2	112	52.9		55.0	
Stage	Ⅱ	63	55.1	0.007	58.0	0.007
	Ⅲ	364	52.4		54.4	
Molecular subtype	CIMP-L/0, MSS	382	52.5	0.006	54.7	0.008
	CIMP-L/0, MSI-H	20	52.0		54.6	
	CIMP-H, MSS	14	60.5		63.7	
	CIMP-H, MSI-H	9	51.2		54.1	

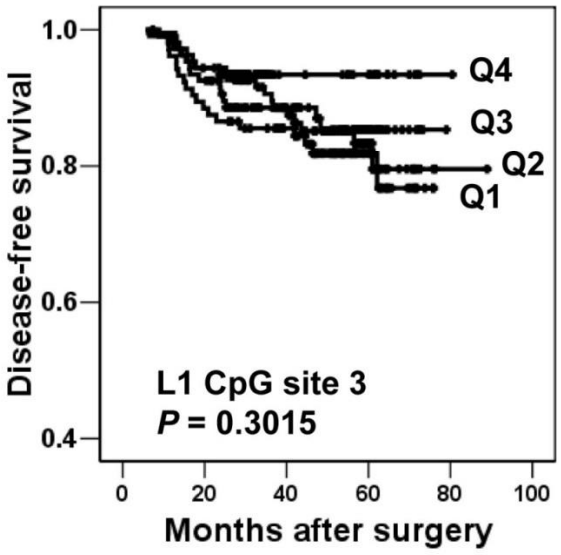

Fig 8. Kaplan-Meier curves of disease-free survival in four quartile groups according to the LINE1 (L1) methylation level on CpG site 3.

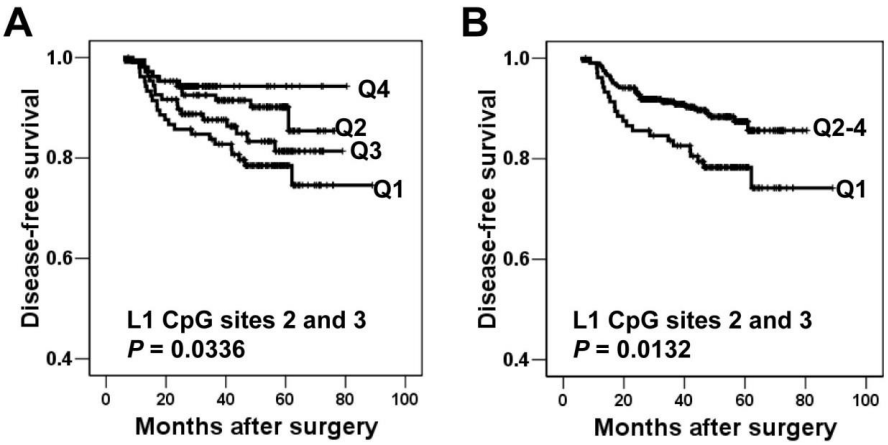

Fig 9. Kaplan-Meier curves of disease-free survival in four quartile groups (Q1, 2, 3 and 4) (A) and in two groups (Q1 vs. Q2-4) (B) according to the LINE1 (L1) methylation level at CpG sites 2 and 3.

The lowest quadrant exhibits the shortest DFS time, while the highest quadrant shows the longest DFS time (A). Three quadrants (Q2, 3, and 4) were grouped into the high L1 methylation group (Q2-4). A significant difference in DFS is noted between Q1 and Q2-4 (B). Q1, 2, 3 and 4 were four quadrants (the lowest 25%, 26-50%, 51-75%, and 76-100%) according to the rank order of the average methylation level of L1 CpG sites 2 and 3.

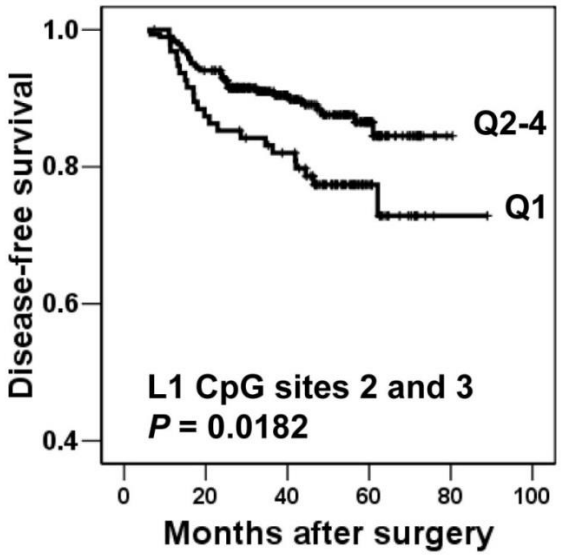

Fig 10. Kaplan-Meier curves of disease-free survival in patients with MSS, CIMP-L/0 CRCs (n= 382).

Q1, 2, 3 and 4 were four quadrants (the lowest 25%, 26-50%, 51-75%, and 76-100%) according to the rank order of the average methylation level of L1 CpG sites 2 and 3.

Table 7. Univariate analysis of overall survival (OS) in 427 patients with colorectal cancer treated with adjuvant FOLFOX

Characteristic	Variable	Number of cases	3- and 5-year OS, %	P value
Age	<60 year	209	88.5 and 84.8	0.8633
	≥60 year	218	90.2 and 84.9	
Tumor subsite	Right colon	139	91.3 and 88.8	0.5539
	Left colon	261	89.0 and 83.8	
	Rectum	27	84.7 and 78.6	

Differentiation	Low	393	89.8 and 85.0	0.2618
	High	34	85.3 and 85.3	
Mucinous histology	Non-mucinous	408	90.1 and 85.4	0.0304
	Mucinous	19	73.7 and 73.7	
TNM Stage	Stage Ⅱ	63	90.2 and 90.2	0.6638
	Stage Ⅲ	364	89.3 and 84.1	
T stage	T1-3	366	90.7 and 86.6	0.0053
	T4	61	81.9 and 74.5	
N stage	N0,1	315	92.5 and 89.2	0.0001
	N2	112	80.8 and 73.2	
Venous invasion	Absent	385	90.7 and 86.6	0.0065
	Present	42	76.8 and 67.9	
Lymphatic emboli	Absent	247	94.7 and 91.4	<0.0001
	Present	180	82.0 and 75.7	
Perineural invasion	Absent	331	91.0 and 88.7	0.0006
	Present	96	83.9 and 67.0	
KRAS	Wild	285	93.2 and 91.5	<0.0001
	Mutant	109	79.9 and 71.0	
BRAF	Wild	399	89.6 and 86.3	0.6801
	Mutant	12	91.7 and 91.7	
MSI	MSS	392	89.5 and 84.9	0.3404
	MSI-H	30	93.3 and 93.30	
CIMP	CIMP-H	404	91.3 and 91.3	0.5259
	CIMP-L/0	23	89.3 and 84.5	
Methylation level of L1 CpG site 3	Q1	105	85.5 and 81.9	0.3015
	Q2	108	90.6 and 83.3	
	Q3	107	88.6 and 85.3	
	Q4	107	93.4 and 93.4	
Methylation level of L1 CpG sites 2 and 3	Q1	106	83.8 and 78.5	0.0336
	Q2	107	92.5 and 90.2	
	Q3	108	87.6 and 81.4	
	Q4	106	94.3 and 94.3	

Table 8. Multivariate analysis of overall survival

Characteristic	Variable	Adjusted HR (95% CI)	P value
L1 methylation (CpG site 2 and 3)			
	Q1	2.000 (1.115-3.585)	0.002
	Q2-4	1	
Mucinous histology			
	Non-mucinous	1	0.014
	Mucinous	3.906 (1.322-11.540)	
T stage			
	T1-3	1	0.043
	T4	1.969 (1.021-3.798)	
N stage			
	N0,1	1	
	N2	2.122 (1.184-3.802)	0.011
KRAS			
	Wild	1	
	Mutant	3.304 (1.888-5.781)	<0.001
Lymphatic emboli			
	Absent	1	
	Present	3.086 (1.602-5.944)	0.001
Perineural invasion			
	Absent	1	
	Present	1.709 (0.933-3.131)	0.082
Venous invasion			
	Absent	1	
	Present	1.216 (0.541-2.701)	0.631

DISCUSSION

In a previous study of Sunami et al., *LINE-1* demethylation occurred early in the colon cancer progression, during adenomatous change of colorectal epithelial cells, and *LINE-1* hypomethylation level did not differ between adenoma and early cancer tissue. (45) Our previous study about DNA methlyation changes in ex-adenoma carcinoma using COBRA assay showed that *Alu* and *LINE-1* methylation levels in adenoma were significantly lower than in adjacent normal mucosa, but similar to cancer samples. (46) Analysis of present study by pyrosequencing assay demonstrated the same result as with previous studies in terms of the time of *LINE-1* demethylation occurrence. A decrease in the methylation level of*LINE-1* and *Alu* was found to occur at the stage of adenoma. However, the step showing initial tumoral hypomethylation was different between *LINE-1* and *Alu*: the low grade dysplasia in *LINE-1* and the high grade dysplasia in *Alu* indicating tumoral *Alu* hypomethylation might occur a little later in the process of tumor progression than that of *LINE-1*. Our present study also showed *Alu* hypomethylation did not differ significantly between tubular adenoma high grade and tubulovillous adenoma

which has higher risk of cancer, in contrast with that *LINE-1* hypomethylation was significantly lower in tubulovillous adenoma than in tubular adenoma high grade. During multistep colorectal carcinogenesis from normal colon to tubular adenoma to colorectal cancer (except for villous or tubular villous adenoma), LINE-1 methylation level showed progressive stepwise decrease with first decrease in the stage of low grade tubular adenoma and second decrease in the stage of carcinoma, while Alu methylation level exhibited a decrease in the stage of high grade tubular adenoma but no further decrease in Alu methylation level.

Tumoral hypomethylation of *LINE-1* and *Alu* did not have prognostic value, when the analysis of the relationship between tumoral *LINE-1* or *Alu* hypomethylation and survival was done for the whole colorectal cancer samples. This result is in contrast to a previous study of Ogino et al. (28) which reported that tumoral *LINE-1* hypomethylation is independently associated with shorter survival among colon cancer patients. However, our analysis limited to the MSI+ CRCs showed that tumoral hypomethylation of *LINE-1* was a poor prognostic

marker. While *LINE-1* methylation levels measured by pyrosequencing in the study of Ogino and colleagues were approximately normally distributed, those of our present study were not. In another study, Ogino and colleagues reported that *LINE-1* extreme hypomethylators whose *LINE-1* methylation level was separate from the remaining normal distribution appeared to constitute a previously-unrecognized, distinct subtype of colorectal cancers. (47) The distribution of *LINE-1* methylation level of 461 cases did not show normal curves and included an extreme hypomethylator group, while that of 207 MSI+ CRC cases was normalized and did not have an extreme hypomethylator group. The presence of the extreme hypomethylator group in the 461 cases available for *LINE-1* methylation analysis in present study might explain the discrepancy between our result and previous study. The separation and more analysis of the extreme hypomethylator group are necessary.

Genetic mutations of the mismatch repair genes MLH1 and MSH2, and also epigenetic inactivation of MLH1, lead to mutations in cancer-related genes and to development of MSI+

CRCs. Regardless of the causal mechanism, MSI+ CRCs have common clinicopathological features, including proximal colon location, frequent mucinous histology, higher density of tumor-infiltrating lymphocytes, and low KRAS mutation frequency.(48) Thus, MSI genotyping may allow identification of discrete molecular subtype of CRCs. Since Thibodeau et al. initially described better survival rates in MSI+ tumors than in MSI-negative (including MSI-L and MSS) tumors, many studies have addressed the prognostic significance of MSI in CRC.(49-51) However, there are still doubts over the clinical utility of MSI as a prognostic marker in CRCs.(52) Although pathologic tumor staging remains a key determinant of prognosis and treatment for MSI + CRC patients, considerable stage-independent outcome variability is observed that likely reflects molecular heterogeneity, underscoring the need for robust prognostic and predictive markers to stratify same-stage tumors as having a "better" or "worse" prognosis.

Although there is still a controversy regarding MSI as a prognostic marker in CRCs, large-scale retrospective studies and meta-analyses have demonstrated that MSI may indeed be

a prognostic marker and help to identify a subgroup of CRCs with better prognoses. However, MSI + CRC is not a homogeneous phenotype because MSI+ CRCs can be composed either of CIMP + or CIMP−negative (CIMP−L and CIMP−0) tumors. Through the previous and present studies, we have found that clinical outcomes of MSI+ CRCs differ depending on CIMP status, and CIMP−H tumors correlate with worse clinical outcomes than those of CIMP−L or CIMP−0 tumors.(41) In the present study, both CIMP−H and *LINE−1*−low were independent prognostic parameters heralding poor prognosis in MSI+ CRCs. Along with the finding of Ogino et al. that a lower LI methylation level is related to poor prognosis of CRCs, our finding suggests that lower *LINE−1* methylation status might serve as prognostic parameter in MSI+ CRCs and thus discriminate favorable and unfavorable clinical outcomes.(28)

In the present study, MSI+ CRCs showed positive correlation between the *LINE−1* methylation level and CIMP status: CIMP−H and CIMP−0 tumors were the highest and lowest in *LINE−1* methylation levels, respectively. Despite the positive correlation between the *LINE−1* methylation level and CIMP

status, the survival analysis yielded contrasting results: for CIMP status, CIMP-H was closely associated with poor prognosis, whereas for *LINE-1* methylation status, *LINE-1*-low was significantly associated with poor prognosis. To explore this seemingly paradoxical relationship, I classified MSI + tumor s into six subgroups according to combinatory statuses of *LINE-1* methylation level and CIMP and performed survival analysis of these six subgroups using the Kaplan-Meier log-rank test. For the same CIMP subtype except for CIMP-0, *LINE-1*-low tumors corresponded with significantly shorter overall survival times than those of *LINE-1*-high tumors, and for the same *LINE-1* methylation status a significant difference of clinical outcomes was noted for CIMP-H, CIMP-L, and CIMP-0 tumors in the order of increasing overall survival time. Along with these results, multivariate analysis using Cox proportional hazard regression models indicated that CIMP status and *LINE-1* status are independent prognostic parameters despite the fact that *LINE-1* methylation levels were higher in CIMP-H tumors than in CIMP-L or CIMP-0 tumors.

A recent study suggested that not only the methylation level but also the methylation pattern of *LINE-1* can be utilized to distinguish cancer cell DNA from normal cell DNA.(29) The Mutirangura group analyzed normal squamous cells and malignant squamous cells for methylation at two CpG sites of the *LINE-1* promoter sequence using combined bisulfite restriction analysis (COBRA), and they classified individual *LINE-1* loci into three patterns: hypermethylated (methylated at both CpG sites), partially methylated (methylated at either CpG site), and hypomethylated (methylated at neither CpG site). The proportion of hypomethylated *LINE-1* loci outperformed the average methylation level of two CpG sites in sensitivity and specificity of cancer detection.(53) So far, however, no study has analyzed the utility of the proportion of hypomethylated *LINE-1* loci in a tumor as a prognostic biomarker. Although the radioisotope-based COBRA assay is highly sensitive and reproducible, the fact that it is labor intensive and involves radioisotopes restricts its clinical application. Because methylation analysis by pyrosequencing cannot provide information regarding methylation at multiple CpG sites in a molecule-based manner, the COBRA assay using

the BioAnalyzer 2100 might be an alternative approach that will be able to provide information about both the amount of methylation at interrogated CpG sites and the proportion of hypomethylated *LINE-1* loci in a large sample set.(54)

Our study has some limitations: we did not perform laser capture microdissection, and thus the measured values of *Alu* or *LINE-1* methylation cannot be considered as the values of the tumor cells themselves. However, we marked and dissected areas where tumor cell density was the highest in individual tumors, and tumor cell proportions in the dissected areas were at least 70 % of the total cells. In the study of Irahara et al., a larger variation of *LINE-1* methylation values was observed in the laser capture microdissection specimens than in the matched manual dissection specimens, indicating the requirement of repeated measurements to minimize the standard deviation of the mean value.(55) Moreover, they demonstrated that there was no substantial difference in the degree of *LINE-1* hypomethylation between macrodissected colon cancers and matched laser capture-dissected specimens, indicating that contaminating normal cells do not substantially affect the *LINE-1* methylation measurement in colon cancer. In

contrast with a macrodissection-based study that did not show any significant associations of *LINE-1* methylation with cancer stage, the microdissection-based study of Sunami et al. demonstrated progressive demethylation of *LINE-1* in relation to TNM stage progression.(30, 45)

FFPE tissue samples stored in pathology departments represent a major source of patient specimens, and retrospective analysis of archival tissue samples enables the correlation of molecular findings with treatment response and clinical outcomes. However, the quality of FFPE tissues depends on factors such as ischemic time before fixation, duration of fixation, or storage conditions, which vary greatly from one sample to another. Most of the studies addressing correlations between tumoral L1 hypomethylation and poor clinical outcome of CRC patients did not use fresh tissue samples but used FFPE tissue samples (56, 57). A recent study has raised concern over the use of FFPE tissue for the assessment of DNA methylation by demonstrating a significant difference of the L1 methylation level in 28% of paired fresh-frozen and FFPE tissue samples (58). In the present study, out

of the four CpG sites that are commonly targeted by studies analyzing the relationship between L1 methylation status of cancer and patient survival, CpG site 3 was found to have the same methylation level between paired fresh-frozen and FFPE tissue samples. Of the two or more serial CpGs, the average methylation level of CpG sites 2 and 3 were not different between paired fresh-frozen and FFPE tissue samples, while the average methylation level of CpG sites 1 to 3 or 1 to 4 were significantly different. Thus, the average methylation level of L1 CpG sites 2 and 3 or methylation level of the L1 CpG site 3 was selected as representative of the overall L1 methylation. However, the average methylation level of L1 CpG sites 2 and 3 was found to be associated with DFS in stage III or high-risk stage II CRCs treated with adjuvant FOLFOX.

In the present study, we found that tumoral L1 hypomethylation (Q1 of L1 methylation at CpG sites 2 and 3) was closely associated with shorter DFS time in a cohort of stage III or high-risk stage II CRC patients who received adjuvant FOLFOX. Correlation between tumoral L1 hypomethylation and shorter survival in patients with colon

cancer or CRC has been demonstrated in previous studies (56). However, these studies did not consider the use of adjuvant chemotherapy in their analysis and thus did not consider the influence of adjuvant chemotherapy on the observed prognostic value of tumoral L1 hypomethylation. A recent study analyzed the prognostic implication of L1 hypomethylation in patients with stage II or III CRC who received surgery alone (n = 54) or postoperative oral fluoropyrimidines (n = 77), and they showed that tumoral L1 hypomethylation is a marker of poor prognosis in CRC patients who received surgery but no adjuvant chemotherapy (59). In contrast to the findings of our study, this recent study reported no difference in survival between adjuvant-treated cancer patients with or without tumoral L1 hypomethylation. The discrepancy might be related to the difference in the methodology of the L1 methylation assay (real time PCR vs. pyrosequencing), the scale of patient population (n = 77 vs. 472), and adjuvant chemotherapy (oral fluoropyrimidines vs. FOLFOX). Of these, the difference in the adjuvant chemotherapy is more likely to explain the discrepancy, and it can be speculated that tumoral L1 hypomethylation might be a prognostic parameter in the

adjuvant setting of FOLFOX but not in the adjuvant setting of fluoropyrimidine alone.

Oxaliplatin in combination with fluoropyrimidines is the current worldwide standard of care for patients with stage III disease because results from three large phase III trials supported the survival benefit of adding oxaliplatin to fluoropyrimidines in the adjuvant setting for patients with stage III colon cancer (36, 60, 61). However, the prognostic potential of tumoral L1 hypomethylation has not been assessed in the adjuvant FOLFOX-treated patients with resectable CRCs. For the first time, our study has demonstrated a correlation between tumoral L1 hypomethylation and shorter DFS time in a cohort of patients with resectable CRCs who were treated with adjuvant FOLFOX. Our previous studies have demonstrated that mucinous histology and KRAS mutation have an independent adverse prognostic impact on stage II or III CRCs treated with adjuvant FOLFOX (39, 40). In the present study, in addition to mucinous histology and KRAS mutation, L1 hypomethylation correlated independently with worse clinical outcome.

Furthermore, because the L1 methylation level was associated with molecular subtypes, we evaluated whether tumoral L1 hypomethylation correlates with shorter DFS in CIMP-negative/MSI-negative CRCs, which comprised 89% of CRC samples in the present study. In the Kaplan-Meier survival analysis, CIMP-/MSI- CRCs with the L1 methylation level of Q1 showed a lower DFS time compared with CIMP-/MSI- CRCs with L1 methylation levels of Q2-4 (Fig.10). Multivariate analysis revealed that low L1 methylation correlated independently with recurrence in patients with CIMP-/MSI- CRC.

Although the present study has demonstrated a close association between low methylation status of *LINE-1* and worse clinical outcomes of stage III or high-risk stage II CRC patients treated with adjuvant FOLFOX, the molecular biological mechanism that links low *LINE-1* methylation status to aggressive CRC behavior is unclear. Three aspects can be considered. First, genomic demethylation, represented by low *LINE-1* methylation status, might lead to chromosomal instability. Genomic demethylation might lead to overexpression

of satellite DNA sequences which might induce chromosomal instability. Chromosomal instability is accompanied by copy number gain or amplification of proto-oncogenes, the generation of fusion genes, and copy number loss of tumor suppressor genes, which might contribute to aggressive tumor cell behavior. Second, decreased methylation of *LINE-1* sequences might lead to aberrant overexpression of proto-oncogenes or decreased expression of genes harboring *LINE-1* sequences in their intronic sequences . Third, because a small proportion of *LINE-1* sequences are capable of retrotransposition, which can cause genome-wide mutations, demethylation induced activation of *LINE-1* sequences might lead to genomic instability. Nevertheless, the mechanism that connects low *LINE-1* methylation status to aggressive tumor cell behavior remains unclear and needs to be fully explored.

In conclusion, our study clearly demonstrates that clinical outcomes of MSI+ CRCs differ depending on their *LINE-1* methylation status and CIMP statuses which are independent prognostic markers within MSI+ CRCs. Information about these

two molecular parameters may help to identify stage II patients that will benefit from postoperative chemotherapy.

Because L1 methylation level at combined two CpG sites (CpG site 2 and 3) are similar between paired fresh-frozen and FFPE tissue samples, combined methylation level of L1 CpG sites 2 and 3 in FFPE tissue samples are likely to represent combined methylation level of L1 CpG sites 2 and 3 in fresh tissue samples. In our present study, tumoral L1 hypomethylation (at CpG sites 2 and 3) was found to be an independent prognostic parameter heralding poor prognosis in patients with stage III or high-risk stage II CRCs treated with adjuvant FOLFOX. Tumoral L1 hypomethylation could be utilized as biomarker for identifying a subset of stage III or high-risk stage CRCs which might be resistant against adjuvant FOLFOX. Further validation of these findings is warranted.

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국문 초록

서론: 유전학적, 후성유전학적 경로를 포함하여 대장암의 발암기전에 대해 종합적으로 이해하기 위해 많은 연구들이 최근 이루어 지고 있다. 후성유전학적 경로에 대한 연구가 이전에 비해 활발히 이루어 지고 있긴 하지만 대장직장암에서 global DNA hypomethylation 에 대한 연구는 아직 부족하다.

암종에서 유전체의 hypomethlyation 은 인간 유전체의 45% 를 차지하는 repetitive DNA element 의 hypomethylation 과 연관이 있다. 종양형성과 관련하여 repetitive DNA element 의 CpG site 가 demethylation 되면 전체 유전체의 hypomethylation 이 유발되고, 결과적으로 유전체의 불안정성, transposable element 와 oncogene 의 활성화를 유발한다. 대표적인 repetitive DNA element 인 Long interspersed nuclear element-1 (*LINE-1*) 의 hypomethylation level 이 높을수록 대장직장암의 예후가 좋지 않다는 보고들이 최근 이루어지고 있다.

이번 연구에서 한국인의 대장직암과 그 전암성 병변에서의 *LINE-1* 과 *Alu* 의 methylation level 에 대해서 분석해 보았다. 특히 Microsatellite instability 양성 (MSI+) 대장암과 FOLFOX aduvant chemotherapy 를 시행한 Stage III 또는 high risk Stage II 에서 임상적 예후와의 관련성에 대해서 밝혔다.

방법: 767 례의 대장직장암, 그 중 207 례의 MSI+ 대장직장암, 154 례의 전암성병변을 대상으로 *LINE-1* 과 *Alu* 의 암종 및 전암성 병변에서의 methylation level 변화를 pyrosequencing assay 를 이용하여 분석하고, 생존자료를 포함하여 여러 임상병리학적 인자와의 연관성에 대해서 분석하였다. 또한 427 례의 stage III, high risk stage II 대장직장암에서의 *LINE-1* methylation status, CpG island methylator phenotype (CIMP), MSI, and *KRAS*/*BRAF* mutation status 를 pyrosequencing assay 를 통해서 분석하였다.

결과: 전체 대장암을 대상으로 분석했을 때는LINE-1 과 Alu 의 hypomethylation 이 예후인자적 가치를 가지지 않았다. 하지만, MSI+ 대장암에서는 단변량 생존 분석에서 low *Alu* methylation 상태 (<18.60%, log-rank test, $P = 0.009$) 와 low *LINE-1* methylation 상태 (<53.00%, log-rank test, $P < 0.001$) 가 통계적으로 유의하게 낮은 생존기간과 연관성을 보였다. MSI+ 대장직장암에서 *Alu* methylation 상태, *LINE-1* methylation 상태, 나이, 병기, 분화, Crohn-like lymphoid reaction, KRAS/BRAF mutation 상태, CpG island methylator phenotype (CIMP)상태, 그리고 peritumoral lymphocytic infiltration 의 9개의 변수를 포함한 다변량 분석에서는 low *LINE-1* methylation 상태만이 통계적으로 유의한 독립적인 예후 인자로 밝혀졌다. ($P = 0.009$),

FOLFOX 보조항암화학요법을 시행한 stage III 또는 high-risk

stage II 대장직장암 에서는 *LINE-1* 의 hypomethylation 은 림프절 전이와 밀접한 연관성을 가졌지만, 발병나이, 성별, 암종의 위치, 분화, mucinous histology, lymphatic emboli, venous invasion, perineural invasion, T stage, and *KRAS/BRAF* mutation은 연관성이 없었다. 다변량 분석을 통해서는 mucinous histology, T stage, N stage, lymphatic emboli, and KRAS mutation 뿐만 아니라, *LINE-1* hypomethylation 이 독립적인 예후인자임을 밝혔다.

결론: MSI 양성 대장직장암의 임상적 결과는 *LINE-1* methylation 상태에 의존하는데, 이는 lower *LINE-1* methylation 상태가 MSI 양성 대장암에서 유의하게 좋지 않은 예후인자임을 시사한다.

FOLFOX 보조항암화학요법을 시행한 stage III 또는 high-risk stage II 대장직장암 환자에서도 암의 *LINE-1* hypomethylation 이 유의하게 독립적으로 좋지 않은 예후 인자로 밝혀져 FOLFOX 보조항암요법에 저항성을 가질 수 있는 군을 선별하는 biomarker 로 사용될 수도 있을 것이다.

주요어 : 대장직장암, 보조항암요법, 예후, DNA methylation, Long interspersed element-1 (*LINE-1*), *Alu*, MSI (Microsatellite instability), pyrosequencing

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