이학석사 학위논문

Methylation status of long interspersed element-1 in advanced gastric cancer and its prognostic implication.

진행 위암에서의 LINE-1의 메틸화 상태 및 메틸화가 예후에 미치는 영향에 대한 연구

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Methylation status of long interspersed element-1 in advanced gastric cancer and its prognostic implication.

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ABSTRACT

Reportedly, pyrosequencing methylation assay might produce inconsistent results between paired snap-frozen and formalin-fixed paraffin embedded archival tissue samples. In this study, we assayed methylation level of four individual CpG sites of L1 using pyrosequencing and found that methylation level of CpG site 3 alone was not different between paired snap-frozen and formalin-fixed paraffin-embedded tissue samples. We aimed to determine whether low methylation status of L1 in CpG site 3 is associated with prognosis of gastric cancer patients.

We analyzed 434 formalin-fixed paraffin-embedded tissue samples of advanced gastric cancer for their methylation status in L1 using pyrosequencing and correlated L1 methylation level with their clinicopathological features.

Older age at onset, male, tumor location at antrum or lower body, intestinal type, and lymphatic or venous invasion were associated with lower methylation level of L1 in CpG site 3 (nucleotide position, 318 of X58075). Methylation level of L1 in CpG site 3 was significantly lower in microsatellite-stable and EBV-negative gastric cancers than in EBV-positive or microsatellite instability-high gastric cancers. Low methylation status of L1 correlated independently with shorter overall survival and disease-free survival time.

Our findings indicate that discrepancy in the methylation level of L1 between fresh tissue and formalin-fixed paraffin-embedded tissue samples depends on CpG sites and that methylation status of L1 CpG site 3 could be utilized as prognositic parameter in advanced gastric cancer.

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INTRODUCTION

DNA methyltransferases methylate cytosines in the context of CpG dinucleotides and 75% to 80% of CpG dinucleotides are methylated in normal human cells (1). CpG sites located in promoter CpG islands are traditionally thought to be unmethylated, whereas CpG sites located in repetitive DNA elements are heavily methylated in normal cells. Promoter CpG island hypermethylation is a common finding in human cancers and generally associated with inactive state of the corresponding gene. Although hundreds of genes can undergo hypermethylation in their promoter CpG island loci in association with cancerization (2), overall content of methylcytosine generally decreases during the malignant transformation from normal cells to cancer cells because of demethylation of CpG sites located in repetitive DNA elements, including long interspersed element-1 (LINE-1 or L1) and Alu (3, 4). Approximately 500,000 copies of L1 are dispersed in human genome comprising about 17% of the human genome and a full-length L1 is about 6kb in length (5). L1 has a high density of CpGs in its initial 460bp region, where cytosine methylation is maintained with high efficiency (6). L1 methylation level has been shown to decrease in gastric epithelial cells with the progression of the lesion along the multistep gastric carcinogenesis although there are wide variations of L1 methylation levels in gastric cancers (GCs) (7, 8). Low L1 methylation status has been demonstrated to be closely

associated with shorter survival of the cancer patients, not only for GCs (7, 8) but also colon cancers (9), rectal cancers (10), esophageal squamous cell carcinomas (11), and lung adenocarcinomas (12, 13).

In the literature, there are two studies available which have explored the relationships between L1 methylation levels of GC tissue samples and clinical outcome of cancer patients and consistently demonstrated close associations of low L1 methylation status with poor clinical outcomes (7, 8). These two studies have limitations in that they used formalin-fixed paraffin-embedded (FFPE) archival tissue samples from a small scale of GC samples (n=193 and 203). However, a recent study has raised a concern over the feasibility of analyzing DNA methylation from DNA obtained from FFPE tissues (14); Toumier el al. analyzed matched fresh-frozen and FFPE samples from 40 colon adenocarcinomas for their methylation status in L1 using pyrosequencing and displayed significant differences of L1 methylation levels in 28% of matched samples (14). This finding has cast a doubt on whether findings of previous studies, close associations between low L1 methylation status and poor clinical outcome of GC patients, are reproducible (7).

In the present studiy, we collected couples of matched snap-frozen and FFPE tissue samples (n=20) and analyzed their methylation status in L1 using pyrosequencing. Through this comparison, we found that, of the four serial CpG sites assayed in our previous study (7), CpG site 3 showed no difference in the methylation level between paired snap-

frozen and FFPE tissue samples. Then we analyzed a large collection of archival tissue samples of advanced GC for their methylation status in L1(CpG site 3) and correlated L1 methylation levels with clinicopathological features, including clinical outcomes.

MATERIALS AND METHODS

Tissue samples

The study was approved by the Institutional Review Board of Seoul National University College of Medicine. A consecutive series of advanced GC cases(T2-T4) was retrieved from the surgical files of the Dep. Of Pathology, Seoul National University Hospital, Seoul, Korea. Among the patients who underwent surgery and extended lymph node dissection (D2) for advanced GC between January 2007 and December 2008, only the patients (n=434) who had data for microsatellite instability (MSI), Epstein-Barr virus (EBV), and L1 methylation status were included in this study. Patients who had a history of other primary malignancies within 5 years or received chemotherapy before surgical resection were excluded. The age of the patients ranged from 23 to 86 years (medina, 61 years) and the male to female ratio was 1.95:1. The following pathological parameters were evaluated through gross and microscopic examinations: tumor differentiation, histological type (Lauren's classification), lymphatic invasion, perineural invasion, venous invasion, and TNM stage (American Joint Committee on Cancer, 7^{th} edition). For multiple synchronous tumors, data were derived from the high-stage tumor or the larger tumor if the synchronous tumors were of the same stage. Through microscopic examination, areas $\sim 1cm^2$ where the tumor cells

were the densest and represented the most prevalent histologic type of the individual case were marked and scraped from the tissue glass slide with a knife. In order to determine the suitability of FFPE tissues for methylation analysis, L1 methylation level was assessed for paired snap-frozen and FFPE tissue samples, including 10 normal lymph nodes, 5 colonic cancers, and 5 breast cancers.

Pyrosequencing methylation analysis

The scraped tissue was collected into microtubes containing 50 μL of tissue lysis buffer and proteinase K. The tubes were incubated for 24 to 48 hours at 55℃ until the tissue-containing buffer fluid turned clear. Proteinase K was inactivated by incubation at 95℃ for 10 min. Following centrifugation, the supernatants were transferred into a newly labeled microtube. DNA samples were bisulfite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). The modified DNA samples were analyzed for their methylation status in L1 using pyrosequencing. In brief, the modified DNA samples were PCR-amplified with the same oligonucleotide primers which were designed by the Issa group for pyrosequencing (15). These oligonucleotide primers were designed toward a consensus L1 sequence, which allowed amplification of a representative pool of L1. L1 elements were amplified using 200 M each of forward primer 5'-TTTTGAGTTAGGTGTGGGATATA-3' and reverse biotinylated primer 5'-AAAATCAAAAAATTCCCTTTC-3' in a 50 μL reaction volume

containing 2.5 ng of bisulfite-treated DNA, 1× CoralLoad PCR Buffer (contains 1.5 mM $MgCl^2$), final 2 mM $MgCl^2$ and 1.5 U of HotStarTaq Plus DNA polymerase (Qiagen, Valencia, CA, USA). PCR thermal amplification profile consisted of an initial denaturation step of 5 min at 95°C, followed by 50 cycles of 30 s at 94°C, 40 s at 57°C, and 40 s at 72°C. The PCR product was purified using Streptavidin Sepharose High Performance beads (GE Healthcare Bio-Sciences Corp., Sweden) and denatured using Denaturation buffer (Qiagen). Next, 0.3 μmol/L of the sequencing primer (5'-AGTTAGGTGTGGGATATAGT-3') was annealed to the purified single-stranded PCR product and the Pyrosequencing reaction was performed using the PyoMark Q24 platform (Qiagen). The level of methylation for each of the 4 analyzed CpG sites (CpG sites 1, 2, 3, and 4, nucleotide positions 328, 321, 318, and 306, respectively (GenBank accession number X58075)) was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines.

MSI determination

The MSI status was determined using the NCI Bethesda recommended microsatellite markers (BAT-25, BAT-26, D2S123, D5S346, and D17S250). GCs with high level of MSI (at least 40% of markers positive) were considered MSI-high (MSI-H) whereas GCs with low level or no MSI (<40% of markers positive) were considered MSS.

EBV-encoded RNA in situ hybridization

The presence of EBV in cancer cells was detected by in site hybridization for EBV-encoded RNA as described previously (16).

Statistical analysis

Statistical analyses were performed using SPSS software for windows, version 21.0 (IBM, Armonk, NY, USA). Two-sided p values of less than 0.05 were considered statistically significant. Because data of L1 methylation level did not follow the normal distribution, mean values between two groups or between three or more groups were compared using Mann-Whitney U test and Kruskal-Wallis test, respectively. Pearson correlation test was used to evaluate the correlation of L1 methylation level between paired snap-frozen and FFPE tissue samples. The clinical database was last updated in January 2014. Disase-free survival (DFS) was calculated from the date of resection of advanced GC to the first date of documented recurrence or the date of death from any cause. Overall survival (OS) was measured from the date of operation to the date of death or the last clinical follow-up time. The average follow-up time (from surgery to death or the last follow-up) was 51.7 months (range, 0.7-85.0 months). Data from patients who were free of recurrence were censored at the date of the last follow-up visit for DFS. OS and DFS were calculated by the Kaplan-Meier method, and comparisons were made by the log-rank test. Hazard ratios were calculated by the Cox proportional hazard model, and

baseline characteristics were adjusted by using backward stepwise model including covariates that have a prognostic value: age, tumor location within the stomach, lymphatic invasion, venous invasion, perineural invasion, TNM stage, and molecular subtype (EBV and MSI status).

RESULTS

Comparison of L1 methylation level between paired snap-frozen and FFPE tissue samples

Through pyrosequencing, we measured methylation levels of four individual CpG sites and compared them between paired snap-frozen and FFPE tissue samples. Although methylation levels of individual CpG sites exhibited strong correlations between paired snap-frozen and FFPE tissue samples (Fig. 1), methylation levels of individual CpG sites 1,2, and 4 except for CpG site 3 were significantly different between paired snap-frozen and FFPE tissue samples (Fig. 2). Regardless of whether DNA samples were obtained from fresh of FFPE tissue samples, methylation level of CpG site 3 was closely correlated with average methylation level of the other three CpG sites (1, 2, and 4) (Fig. 3). Thus, we determined to use methylation level of CpG site 3 as a representative value of L1 methylation level for FFPE tissue samples.

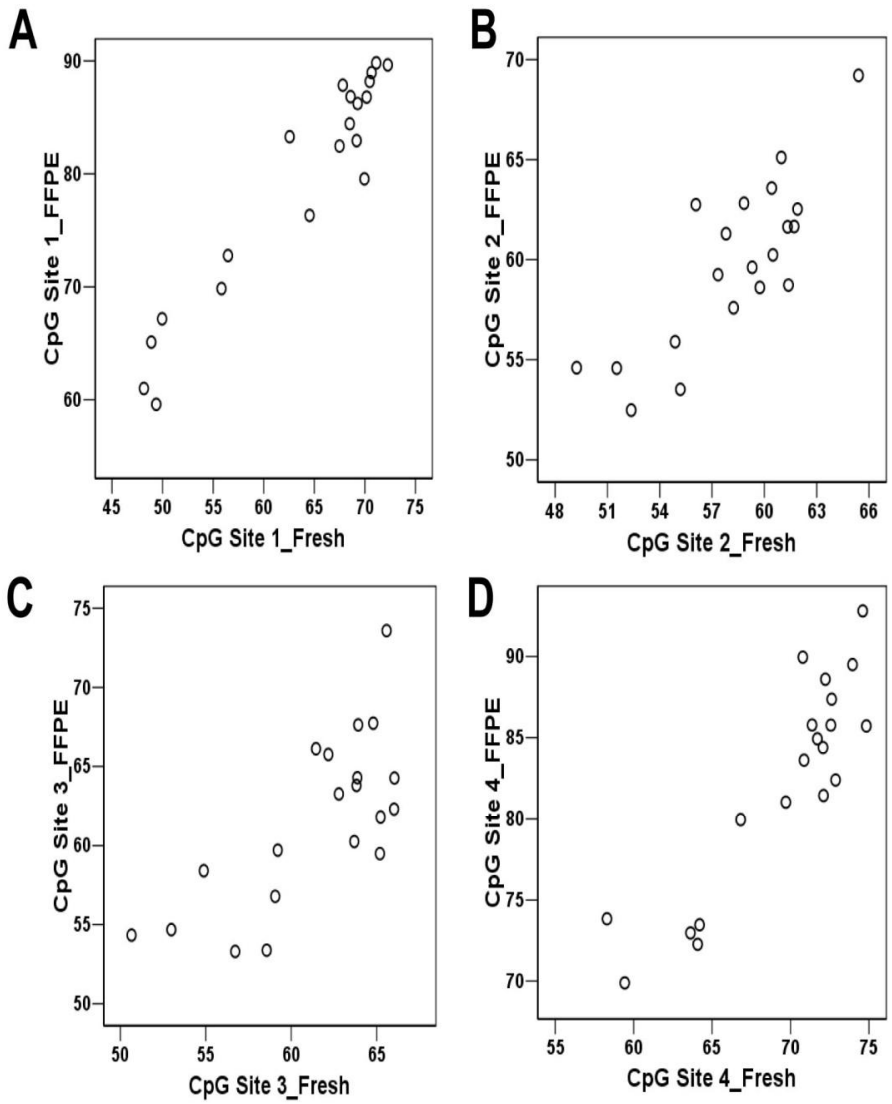

Figure 1. Correlation of methylation levels in L1 CpG sites.

Pyrosequencing methylation assay were performed in L1 CpG sites 1 (A), 2 (B), 3 (C), and 4 (D) between paired fresh-frozen and FFPE tissue samples. The values of spearman' rho were 0.899, 0.699, 0.801, and 0.759.

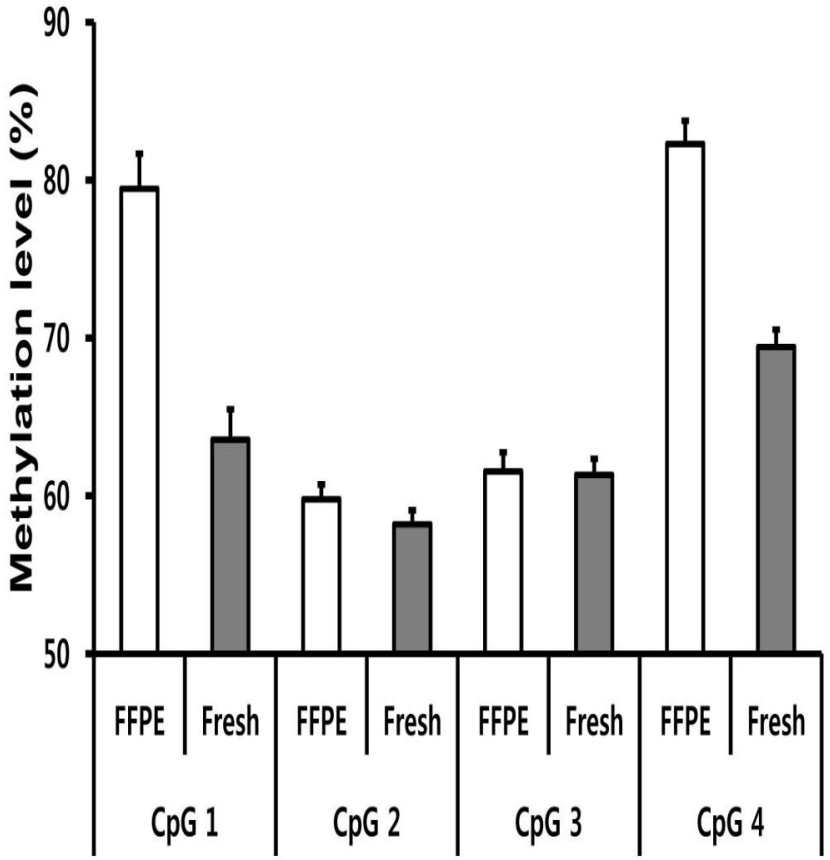

Figure 2. Comparison of methylation levels in individual CpG sites.

Comparison of methylation levels in individual CpG sites between paired snap-frozen and formalin-fixed, paraffin-embedded (FFPE) tissue samples. Methylation level was significantly different in CpG sites 1, 2, and 4 except for CpG site 3 between paired snap-frozen and FFPE tissue samples.

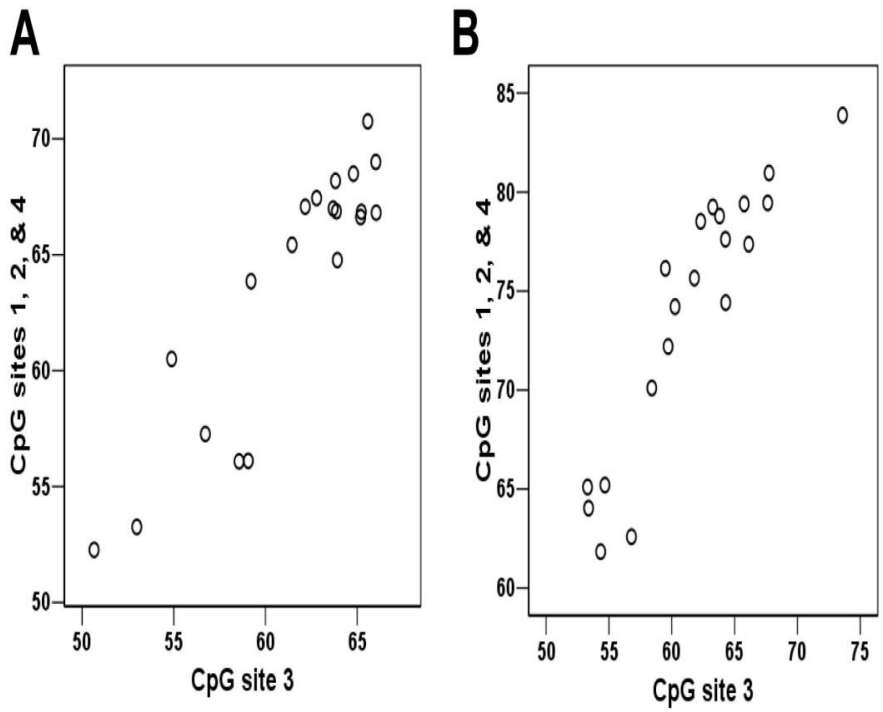

Figure 3. Correlation of methylation level of CpG site 3 with other CpG sites in fresh frozen tissue samples.

Correlation of methylation level of CpG site 3 with methylation levels of CpG sites 1, 2, and 4 in fresh frozen tissue samples (A) and FFPE tissue samples (B). Spearman's rho was 0.899 (A) and 0.762 (B).

Relationship between L1 methylation level and clinicopatholofical features

Methylation level of L1 showed close associations with age, gender, tumor location within the stomach, histologic type (Lauren's classification), lymphatic emboli, venous invasion, and molecular subtype(Table 1). Methylation level of L1 in advanced GCs was higher in younger age than in older age at onset, in female than in male, in GCs involving high body than in GCs not involving high body, in diffuse-type or mixed-type than in intestinal-type, in EBV-positice or MSI-H than in MSS/non-EBV type, in GCs with no lymphatic emboli than in GCs with lymphatic emboli, and in GC with no venous invasion than in GC with venous invasion. L1 methylation level did not correlate with perineural invasion or cancer staging.

Table 1. Relationships between L1 methylation level and clinicopathological parameters of gastric cancer patients.

Characteristic	Variable	No. of cases	methylation level (CpG site 3), %	P-value
Age	<61 year	210	69.4	0.038
	≥61 year	224	67.1	
Sex	Male	287	67.4	0.009
	Female	147	70.0	
Tumor subsite	Not involving high body	311	67.5	0.021
	Involving high body	123	70.2	
Stage	I	53	69.0	0.353
	II	148	69.1	
	III	184	67.8	
	IV	49	66.8	
T stage	T2	99	67.5	0.764
	T3	167	68.3	
	T4	152	68.6	
N stage	N0	131	70.1	0.088
	N1	84	68.1	
	N2	81	67.4	
	N3	138	67.1	
M stage	M0	385	68.4	0.153
	M1	49	66.8	
Lauren classification	Intestinal	163	64.7	<0.001
	Diffuse	206	70.9	
	Mixed	58	69.0	
	Unclassified	7	67.2	
Venous invasion	Absent	325	69.4	<0.001
	Present	109	64.9	
Lymphatic emboli	Absent	163	69.9	0.006
	Present	271	67.3	
Perineural invasion	Absent	196	67.2	0.312
	Present	238	69.1	
Molecular subtype	EBV-positive	33	74.2	<0.001
	MSI-H	52	72.0	
	MSS/non-EBV	347	67.2	

L1 methylation level and patient survival

Patient survival was followed-up in 492 patients since two patients were lost during the follow-up. Through performing univariate analysis with serial cut-off values from the lowest to the highest L1 methylation level, we found that cut-off value set at 55.7% for CpG site generated the greatest prognostic value in both overall survival (OS) and disease-free survival (DFS). Low methylation status of L1 (<55.7%) was closely associated with overall survival (OS) time and disease-fress survival (DFS) time (*P*=0.0001 and *P*=0.0001, respectively) (Fig. 4 a & b). Through Kaplan-Meier log rank test, we found that older age at onset, tumor location in high body, higher cancer stage, venous invasion, lymphatic invasion, and perineural invasion were closely associated with shorter OS and DFS time (Table 2 and Table 3). Molecular subtype (EBV-positive, MSI-H, and MSS/non-EBV) was closely associated with DFS but not with OS. When incorporated into the multivariate models, low methylation status of L1 correlated independently with OS(HR =2.009; 95% CI, 1.324-3.048) (Table 4) and DFS (HR=2.153; 95% CI, 1.430-3.243) (Table 5).

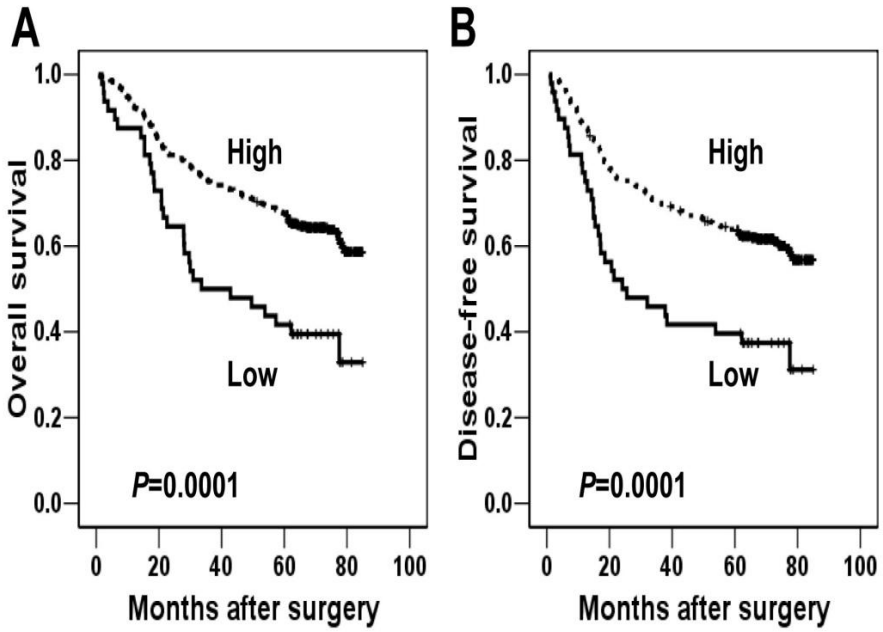

Figure 4. Kaplan-Meier survival analysis with log-rank test.

Low methylation status (<55.7%) of L1 CpG site 3 was closely associated with shorter overall survival (A) and disease-free survival time (B).

Table 2. Univariate analysis of overall survival (OS) in 432 patients with advanced gastric cancer

Characteristic	Variable	Number of cases	3- and 5-year OS, %	P-value
Age	<61 year	210	75.2 and 70.5	0.0323
	≥61 year	222	69.4 and 59.4	
Tumor subsite	Not involving high body	310	77.4 and 71.9	<0.0001
	Involving high body	122	59.0 and 46.7	
Stage	I	52	94.2 and 84.6	<0.0001
	II	148	89.9 and 83.8	
	III	183	65.0 and 56.8	
	IV	49	22.5 and 16.3	
Lauren classification	Intestinal	163	76.7 and 67.5	0.2694
	Diffuse	205	66.7 and 61.3	
	Mixed	58	81.0 and 70.7	
	Unclassified	7	57.1 and 57.1	
Venous invasion	Absent	325	78.8 and 70.5	<0.0001
	Present	107	52.3 and 47.7	
Lymphatic emboli	Absent	163	88.3 and 82.2	<0.0001
	Present	270	62.5 and 54.3	
Perineural invasion	Absent	195	80.5 and 71.8	0.0009
	Present	237	65.4 and 59.1	
Molecular subtype	EBV-positive	33	75.8 and 66.7	0.1391
	MSI-H	51	82.4 and 76.4	
	MSS/non-EBV	347	70.2 and 63.0	
Methylation level of L1 CpG site 3	Low (<55.7%)	48	50.0 and 41.7	0.0001
	High (>55.7%)	384	75.0 and 67.7	
	High (>63.0%)	342	76.0 and 68.4	

Table 3. Univariate analysis of disease-free survival (DFS) in 432 patients with advanced gastric cancer

Characteristic	Variable	Number of cases	3- and 5-year DFS, %	P-value
Age	<61 year	210	69.4 and 67.0	0.0539
	≥61 year	222	65.3 and 55.7	
Tumor subsite	Not involving high body	310	74.2 and 69.0	<0.0001
	Involving high body	122	49.7 and 41.4	
Stage	I	52	90.4 and 84.6	<0.0001
	II	148	87.2 and 81.1	
	III	183	57.1 and 51.1	
	IV	49	18.4 and 14.3	
Lauren clssification	Intestinal	163	72.8 and 64.6	0.4031
	Diffuse	204	61.5 and 57.0	
	Mixed	58	74.1 and 67.1	
	Unclassified	7	57.1 and 57.1	
Venous invasion	Absent	325	72.8 and 66.5	<0.0001
	Present	107	50.9 and 45.4	
Lymphatic emboli	Absent	163	84.0 and 79.6	<0.0001
	Present	269	57.6 and 50.2	
Perineural invasion	Absent	1965	77.9 and 68.5	0.0004
	Present	237	59.1 and 55.2	
Molecular subtype	EBV-positive	33	72.0 and 68.9	0.0389
	MSI-high	51	80.4 and 72.6	
	EBV-negative and MSS/MSI-low	346	65.3 and 58.9	
Methylation level of L1 CpG site 3	Low (<55.7%)	48	46.9 and 40.5	0.0001
	High (>55.7%)	384	70.0 and 63.9	

Table 4. Multivariate analysis of overall survival (L1 methylation at CpG site 3)

Characteristic	Variable	Adjusted HR (95% CI)	P-value
L1 methylation (CpG site 3)			
	Low	2.009 (1.324-3.048)	0.001
	High	1	
Age			
	Low	1	<0.001
	High	1.853 (1.332-2.578)	
Tumor subsite			
	Not involving high body	1	<0.001
	Involving high body	1.899 (1.383-2.606)	
Cancer stage			<0.001
	Stage I	1	
	Stage II	1.100 (0.522-2.317)	0.802
	Stage III	2.275 (1.112-4.652)	0.024
	Stage IV	8.295 (3.852-17.862)	<0.001
Lymphatic emboli			
	Absent	1	
	Present	1.510 (1.019-2.237)	0.040
Perineural invasion			
	Absent	1	
	Present	1.386 (0.984-1.954)	0.062

Table 5. Multivariate analysis of disease-free survival (L1 methylation at CpG site 3)

Characteristic	Variable	Adjusted HR (95% CI)	P-value
L1 methylation (CpG site 3)			
	Low	2.153 (1.430-3.243)	<0.001
	High	1	
Age			
	Low	1	
	High	1.691 (1.235-2.316)	0.001
Tumor subsite			
	Not involving high body	1	
	Involving high body	1.804 (1.326-2.453)	<0.001
Cancer stage			
	Stage I	1	
	Stage II	1.404 (0.649-3.037)	0.389
	Stage III	3.136 (1.488-6.607)	0.003
	Stage IV	8.742 (3.976-19.221)	<0.001
Lymphatic emboli			
	Absent	1	
	Present	1.509 (1.030-2.211)	0.035
Perineural invasion			
	Absent	1	
	Present	1.476 (0.985-1.922)	0.061

DISCUSSION

In consistent with a previous study (14), our study has demonstrated a significant difference in L1 methylation level (average methylation level of a total of four CpG sites) between paired fresh-frozen and FFPE tissue samples although methylation levels of four individual CpG sites were closely correlated between paired fresh-frozen and FFPE tissue samples. However, of the four CpG sites assayed by pyrosequencing, methylation level of CpG site 3 was not different between paired snap-frozen and FFPE tissue samples. Thus, we considered methylation level of L1 CpG site 3 in FFPE tissue samples as representing methylation level of L1 CpG site 3 in fresh tissue samples. The present study demonstrated that low methylation status at L1 CpG site 3 was associated with older age at onset, male, tumor location at lower body or antrum, intestinal type, lymphatic or venous invasion, and MSS/non-EBV molecular subtype. Multivariate analysis revealed that low methylation status at L1 CpG site 3 was an independent prognostic parameter heralding poor prognosis in patients with advanced GC.

Because the present study did not conduct laser capture microdissection, a concern may well be raised over whether infiltrating immune and stromal cells may influence the analysis of L1 methylation level in GC tumor samples (4, 17). After we estimated the ratio of

tumor cell to non-neoplastic cell in tumor areas which were marked for manual dissection, we grouped GC cases into two subsets according to tumor purity (<50% (n=211), and, ≥ 50% (n=221)) and then evaluated prognostic potential of low methylation status of L1 CpG site 3 in each subsets. Prognostic significance of low methylation status of L1 CpG site 3 was not seen in a subset with tumor purity < 50% (Fig. 5) but observed in subsets of tumor purity ≥50% (Fig. 6). On multivariate analyses restricted to GCs with tumor purity ≥50% (n=221), low methylation status of L1 CpG site 3 was an independent prognostic factor in both aspects of OS and DFS (Table 6 & 7). These findings suggest that infiltrating immune and stromal cells might perturb the prognostic significance of L1 methylation status in GCs with low tumor purity. Laser capture microdissection is necessary to enrich tumor cell proportion to elucidate whether low methylation status of L1 is a prognostic factor in GCs with low tumor purity.

In a recent integrative genomic study of GCs (18), GCs were classified into EBV-positive, MSI-H, and MSS/non-EBV tumors which were further subclassified into intestinal-, diffuse-, and mixed-type tumors. This study, using the Illumina HumanMethylation 450K assay, has demonstrated that EBV-positive GCs have the highest degree of genome-wide hypermethylation but minimal degree of genome-wide demethylation, whereas MSI-H GCs have both genome-wide hypermethylation and demethylation. However, high degree of genome-wide demethylation is MSI-H GCs of the study is contrasted

with high methylation level of L1 CpG site 3 in the present study and this apparent discrepancy is related to the fact that the Illumina Human Methylation 450K array does not cover CpG sites located within L1 (19). Further study is required to explore whether MSI-H GCs harbor extensive demethylation in CpG sites located outside CpG island loci and repetitive DNA elements but maintain high methylation state in CpG sites located within repetitive DNA elements. In the present study, EBV-positive GCs showed higher methylation level at L1 CpG site 3 compared with the other molecular subtypes. Because EBV-positive GCs tend to be associated with high amount of immune cell infiltration, it is unclear whether high level of methylation in L1 was related to the high amount of immune cell infiltration. However, when we compared methylation levels of L1 CpG site 3 between EBV-positive GCs with low tumor purity (<50%) and with high tumor purity (≥50%), no difference was found. And furthermore,, with comparison restricted to GCs with tumor purity ≥ 70%, EBV-positive GCs showed higher methylation level of L1 CpG site 3 compared with MSI-H GCs or MSS/EBV-negative GCs. To clarify whether tumor cells of EBV-positive GC have high level of L1 methylation, laser capture microdissection-based study is necessary.

Because L1 methylation status was associated with EBV-positive or MSI-H positive GCs, it might be questioned whether the more favorable outcome in cases with high levels of L1 methylation is simply a relection of the known better outcome for these types of GC. When

we restricted survival analysis to MSS/non-EBV GCs, tumoral L1 hypermethylation was found to be still an independent prognostic parameter heralding poor prognosis in both OS and DFS. Low methylation status of L1 CpG site 3 correlated independently wirh OS (HR = 2.054; 95% CI, 1.324-3.186) and DFS (HR = 1.907; 95% CI, 1.239-2.934).

Although the present study has demonstrated a close association between low methylation status of L1 and worse clinical outcome of GC patients, it is unclear regarding molecular biological mechanisms how low L1 methylation status contributes to the aggressive behavior of GC. Three aspects can be considered : First, genomic demethylatin, represented by low L1 methylation status, might lead to chromosomal instability (20, 21). Genomic demethylation might lead to overexpression of satellite DNA sequences which might induce chromosomal instability (22). Chromosomal instability is accompanied with copy number gain or amplification of proto-oncogenes, generation of fusion genes, and copy number loss of tumor suppressor genes, which might contribute to aggressive behavior of tumor cells. Second, decreased methylation of L1 sequences might lead to aberrant overexpression of proto-oncogenes or decreased expression of genes harboring L1 sequences in their intronic sequences (23-25). Third, Because a small proportion of L1 sequences are capable of retrotransposition which can cause genome-wide mutations, demethylation-induced activation of L1 sequences might lead to

genomic instability. Nevertheless, the mechanism how low L1 methylation status contributes to aggressive behavior of tumor cells remains unclear and to be fully explored.

In conclusion, findings of our present study support those of previous studies which low L1 methylation status is significantly and independently associated with poor prognosis of GC patients. A further study using fresh tissue samples of GC is necessary to confirm the strong association between tumoral L1 hypomethylation and poor prognosis of GC patients.

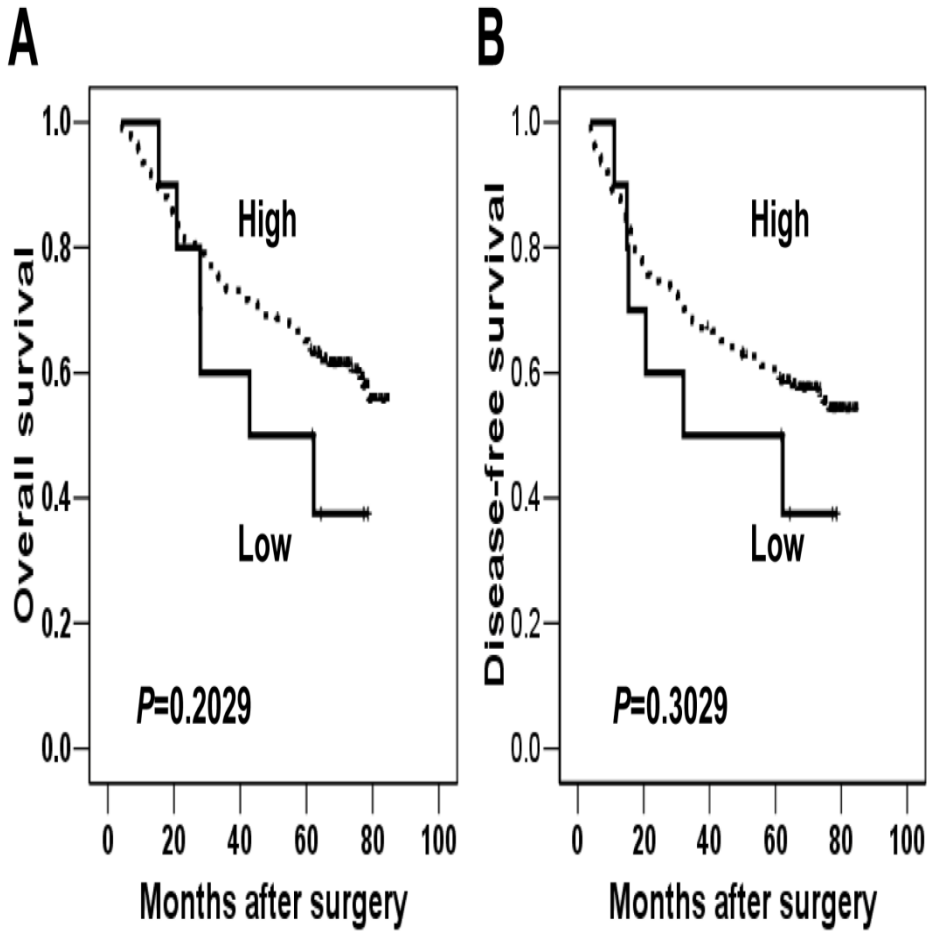

Figure 5. Kaplan-Meier survival analysis with log rank test was performed in advanced GCs with tumor purity < 50%.

No significant difference was noted in overall survival (A) and disease-free survival (B) between advanced gastric cancers with low methylation status (<55.7%) of L1 CpG site 3 and high methylation status (>55.7%)

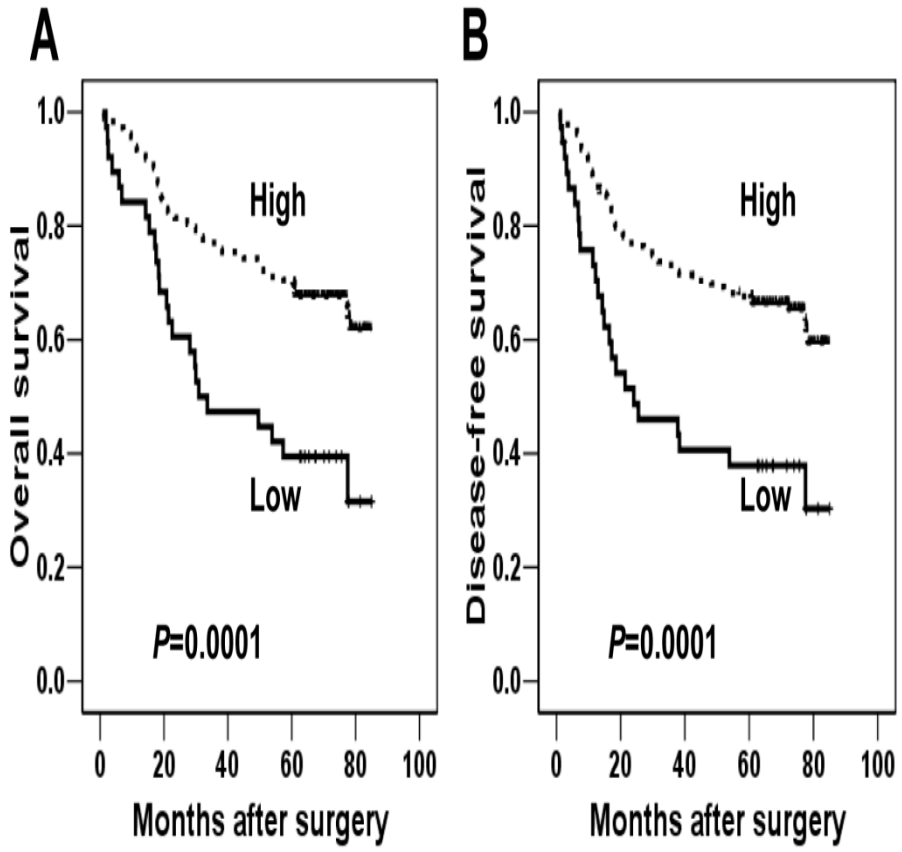

Figure 6. Kaplan-Meier survival analysis with log rank test was performed in advanced GCs with tumor purity ≥ 50%.

Significant difference was noted in overall survival (A) and disease-free survival (B) between advanced gastric cancers with low methylation status (<55.7%) of L1 CpG site 3 and high methylation status (>55.7%).

Table 6. Multivariate analysis of OS (n=221, tumor purity ≥50%)

Characteristic	Variable	Adjusted HR (95% CI)	P-value
Methylation level of L1 CpG 3			
	Low	2.315 (1.403-3.818)	0.001
	High	1	
Age			
	Low	1	
	High	1.528 (0.957-2.438)	0.076
Site			
	Not involving high body	1	
	Involving high body	1.695 (1.057-2.716)	0.028
Cancer stage			<0.001
	Stage I	1	
	Stage II	0.754 (0.275-2.070)	0.584
	Stage III	1.504 (0.578-3.912)	0.403
	Stage IV	6.169 (2.150-17.703)	0.001
Lymphatic emboli			
	Absent	1	
	Present	1.783 (0.949-3.349)	0.072
Perineural invasion			0.151
	Absent	1	
	Present	1.427 (0.879-2.317)	

Table 7. Multivariate analysis of DFS (n=221, tumor purity ≥50%)

Characteristic	Variable	Adjusted HR (95% CI)	P-value
Methylation level of CpG 3			
	Low	2.213 (1.477-3.316)	<0.001
	High	1	
Age			
	Low	1	
	High	1.697 (1.240-2.323)	0.001
Site			
	Not involving high body	1	
	Involving high body	1.793 (1.319-2.437)	<0.001
Cancer stage			<0.001
	Stage I	1	
	Stage II	1.272 (0.609-2.656)	0.522
	Stage III	2.824 (1.392-5.730)	0.004
	Stage IV	7.889 (3.718-16.737)	<0.001
Lymphatic emboli			
	Absent	1	
	Present	1.532 (1.046-2.243)	0.029
Perineural invasion			
	Absent	1	
	Present	1.375 (0.985-1.919)	0.061

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국문 초록

진행 위암에서의 LINE1의 메틸화 상태 및 메틸화가 예후에 미치는 영향에 대한 연구

Pyrosequencing 을 이용한 메틸화 분석을 수행했을 때, 대응되는 급속 냉동시킨 조직 샘플과 파라핀 블록 샘플의 메틸화 상태가 일치하지 않는 것이 보고되고 있다. 본 연구에서는 pyrosequencing 을 이용하여 L1 의 4 개 CpG sites 의 메틸화 정도를 각각 측정하였고, 그 결과 CpG site 3 에서만 급속 냉동시킨 조직 샘플과 파라핀 블록 샘플 사이에서 메틸화 정도의 차이가 나지 않는 것을 확인했다. 우리는 CpG site 3 에서 L1 의 낮은 메틸화 상태가 위암 환자들의 예후와 관련이 있는지 확인하고자 하였다.

우리는 pyrosequencing 을 수행하여 434 개의 진행 위암 파라핀 블록에서 L1 의 메틸화 상태를 분석하였고, L1 메틸화 수치와 임상병리학적인 특성을 연관시켜보았다.

분석 결과를 보았을 때, 늦은 발병 시기, 남성, 암이 위의 전정부에 위치하거나, 장형 위암, 림프관이나 정맥성 침윤이 있을 경우, CpG site 3(nucleotide position, 318of X58075)에서 L1 의 낮은 메틸화와 연관이 있는 것을 확인했다. EBV 양성 또는 microsatellite instability-high 인 위암에서보다 microsatellite-stable 이고 EBV 음성인 위암에서의 CpG site 3 에서 L1 의 메틸화

정도는 상당히 낮게 측정이 되었다. 이와 별개로, L1 에서의 낮은 메틸화는 짧은 전체 생존율과 무병 생존율과도 연관이 있었다.

결론적으로, 본 연구는 정상조직과 파라핀 포매 절편 조직 샘플에서의 L1 메틸화 상태가 일치하지 않는 것은 CpG site 에 달려있고, L1 CpG site 3 에서의 메틸화 상태는 진행 위암에서의 예후 변수로써 활용되어 질 수 있다는 것을 보여준다.

주요어 : Gastric cancer, prognosis, Long interspersed element-1, hypomethylation

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