의학석사 학위논문

Hypomethylation of long interspersed element-1 is a prognostic factor in stage III or high-risk stage II colorectal cancers treated with adjuvant FOLFOX

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이 논문을 의학석사 학위논문으로 제출함.

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Hypomethylation of long interspersed element-1 is a prognostic factor in stage III or high-risk stage II colorectal cancers treated with adjuvant FOLFOX

by
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ABSTRACT

Background: Hypomethylation of Long intersperse element-1 (L1) is considered a surrogate marker for a decrease in methylcytosine content in tumor cells. Tumoral L1 hypomethylation correlates worse clinical outcome in patients with gastric cancer or esophageal cancer. However, it remains unclear whether low L1 methylation is a prognostic marker in colorectal cancers (CRCs). We aimed to elucidate whether tumoral L1 hypomethylation may have a prognostic role in CRCs treated with adjuvant FOLFOX.

Materials and methods: We analyzed a total of 427 resected cases of stage III or high-risk stage II CRC for their statuses in L1 methylation, CpG island methylator phenotype, microsatellite instability, and KRAS/BRAF mutation. L1 methylation was assayed by pyrosequencing.

Results: 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. 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.

Conclusion:Tumoral L1 hypomethylation correlated independently with poor prognosis in patients with resectable CRC treated with adjuvant FOLFOX.

Key word :LINE-1; colorectal cancers ; FOLFOX ; prognostic factor ; hypomethylation

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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 that of the whole cancers is low and five year survival rate is gradually increased from 54.8% to 72.6% from 1993 to 2010.

Early detection of tumor could attribute the cause of survival improvement. However, active research on the tumorigenesis, molecular subtype and prognostic factors of colorectal cancer and its clinical application are thought to be more critical to the survival improvement. The targeted therapy using monoclonal antibody drug rerated to the BRAF, KRAS mutation would be a representative example. [1]

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. [2]

The epigenetic tumorigenesisoccurs through CpG island (promoter CpG region) hypermethylation and global DNA hypomethylation resulting in repression of tumor suppressor genes or induction of genomic instability respectively.[3-6]In this study we focused on the global DNA hypomethylationin the stage III or high-risk stage II colorectal cancers treated with adjuvant FOLFOX chemotherapy, and elucidated the value as a prognostic factor.

Stage III colorectal cancers had been known to cause high recurrence rate, up 50%, and the death due to metastasis.[7]However, the recurrence rate has been reduced since 1990s, and adjuvant chemotherapy using FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin) regimen contributed to decrease recurrence rate and mortality rate. [8] Researches on the prognostic factor of these group of colorectal cancers are actively being done, and it has been recently reported that KRAS mutation and mucinous histology are associated with worse prognosis in stage III or high-risk stage II colorectal cancer patients treated with adjuvant FOLFOX. [9,10],However, the study related to epigenetic tumorigenic mechanism is limited, especially studies to evaluate the value of cancer

genomic hypomethylationas a prognostic factor.

Genomic hypomethylation is related to repetitive DNA element which comprise 55% of human genome. Transposable repetitive DNA element including LINE-1 (long interspersed nuclear element) and Alu, one of the representative example of repetitive element, consist of45% of genome. In human, LINE-1(long interspersed nuclear element) occupies 17% of genome,Alu which is one of the SINE-1(short interspersed nuclear element) occupies 11% of genome. [11]

LINE-1 is a retrotransposon interspersed in genome as 500,000 copies. It is about 5kb long and has CpGisland at 5' untranslated region.Alu is a retrotransposon interspersed as 1,000,000 copies, is 300bp long and also has high CpG concentration.CpGisland in these retrotansposon is methylated in normal cells to repress the transposable activity of itself. [12-14]However, they are hypomethylated in the tumors resulting in causinggenomic instability [4,5] and activation of transposable element[15-17]and oncogene.[18,19]

The methylation status of LINE-1 and Alu has been known to have close correlation with DNA methylation content of whole genome, and therefore it could be used as a surrogate marker

of global DNA methylation biomarker.[20,21] There have been many studies to prove LINE-1 andAlu serve as a meaningful biomarker of epigenetic change in tumors. [18,22-25] For example, it has been shown that LINE-1 hypomethylation is statistically significantly corelated with poor prognosis in gastric cancer, esophageal cancer and glioma. [23-25]However, it remains unclear if low LINE-1 methylation is a prognostic marker in colorectal cancer, even though it has been proved that there is LINE-1 methylation variability and LINE-1 extreme hypomethylation could be classified as one subgroup in colorectal cancer[22], and that LINE-1 hypomethylation is inversely correlated with micorosatellite instability [26,27]. Recently, our group demonstrated that LINE-1 hypomethylation in MSI-high colorectal cancer is significantly correlated with short overall survival [28]. In present study, we tried to verify whether LINE-1 hypomethylationis a poor prognostic factor in stage III and high-risk stage II colorectal cancer treated with FOLFOX adjuvant chemotherapy.

Materials and Methods

Patients and tissue samples

We used formalin-fixed, paraffin-embedded archival tissue blocks of stage III orhigh risk stage II colorectal cancer patients who underwent curative resection between April 2005 and December 2011 and received at least 6 cycles of adjuvant FOLOX(5-fluorouracil, leucovorin and oxaliplatin) chemotherapy at Seoul National University Hospital. Chemotherapy regimen was FOLFOX-4 or modified FOLFOX-6, and 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, biopsy. This cohort consisted of 521 patients has been reported in our previous studies [9,10], and as previously described inclusion criteria other than tumor stage were age over 18 and adenocarcinoma histology. High-risk stage II was defined if patients had any of

the following: T4 lesion, obstruction or perforation, lymphovascular invasion, perineural invasion, or poorly differentiated histology. Exclusion criteria were previous chemotherapy for colorectal cancer, previous radiotherapy for CRC, signet ring cell histology, distant metastasis, and history of other malignancy within 5 years. Of the eligible 521 cases, 427 were available for methylation analysis of LINE-1. Electronic medical records were reviewed for clinicopathological information. This study was reviewed and approved by Institutional Review Board of Seoul National University College of medicine.

Determination of MSI

The microsatellite status of tumors were determined by five NCI markers: BAT25, BAT26, D2S123, D5S346, and D17S250. Samples were classified as MSI-high (MSI-H) when at least two markers showed instability, as MSI-low (MSI-L) when one marker showed instability, and as MSS when all markers

were stable.

KRAS and BRAF Mutation Analysis

The mutation of KRAS codons 12 and 13 and BRAF codon 600 were detected by enriched PCR-restriction fragment length polymorphism and confirmed by an ABI automatic sequencer (Applied Biosystems, Foster City, CA, USA),as described before. [29]

DNA extraction and bisulfite modification

We marked tumor areas of the individual cases and dissected them manually with knife blades. The dissected tissues were lysed with buffer composed of 50 ㎕ tissue lysis buffer (1% Tween 20 (Sigma, St. Louis, MO), 100 mM of Tris-HCl buffer (pH 7.6), 1 mM of EDTA), and 0.4 ㎍/㎕ proteinase K (Sigma)

in 1.5 mL microtubes. These tubes were incubated at 55° C for 24-48h, and then at 95° C for 10 min. [30] Bisulfite conversion of lysed tissue was done using Zymo EZ DNA methylation kit (Zymo Research Co., Orange, CA, USA) according to the manufacturer's protocol.

Analysis of methylation status of LINE-1 by pyrosequencing

Methylation level of LINE-1 was measured bypyrosequencing assay which determine the DNA sequence by primer-directed real time PCR without electrophoresis by PyroMark Q24 system (Biotage AB, Uppasala, Sweden) as described in previous studies. [28,31]In brief, bisulfite-modified DNA samples were amplified by PCR with oligonucleotide primers designed for a consensus sequence for LINE-1, allowing amplification of a pool of repetitive element LINE-1 of which methylation level serves as a surrogate marker of genomic DNA methylation

changes.The primers and PCR conditions are described in supplementary Table 1. The biotinylated PCR products were purified and quantified in the PyroMark Q24 system. The degree of methylation of LINE-1 was analyzed as the percentage of 5' -methylated cytosines over the sum of methylated and unmethylatedcytosines, that is the ratio of C to the sum of C and T at each CpG site was evaluated as percentage. The average of the relative amount of C in the LINE-1CpG sites 2 and 3 was selected as the overall LINE-1 methylation.

Statistical analysis

All statistical analyses were performed using the statistics software Statistical Program for Social Sciences Version 18.0 (SPSS Inc., Chicago, IL, USA). When assessing the differences in mean value of LINE-1 methylation level between two groups we used non-parametric Mann-Whitney U test, and non-parametric Kruskal-Wallis test for the comparison of the mean

values among three or more groups, because LINE methylation levels did not show normal distribution. Clinical data was last updated in January 2014. DFS (disease free survival) was calculated from the date of operation to the date of recurrence or death by Kaplan-Meier analysisusing quartile groups of L1 CpG sites 2 and 3 and the significance of differences among four groups was determined by the log-rank test. We calculated Hazard ratio of death according to LINE-1 methylation using Cox proportional hazards models. All tests were two-sided and statistical significance was set at P values <0.05.

Result

Pyrosequencing assay for L1 methylation status

In our previous study, we analyzed methylation levels of four CpG sites of L1 in paired snap-frozen and formalin-fixed, paraffin-embedded (FFPE) tissue samples (n=20) using pyrosequencing and realized that despite the presence of strong correlations in methylation levels of individual CpG sites between paired snap-frozen and FFPE tissue samples, significant difference existed in methylation level of CpG sites 1, 2, and 4. When we compared methylation levels of any combinations of four CpG sites between paired snap-frozen and FFPE tissue samples, methylation level of CpG site 3 or combined methylation level of CpG sites 2 and 3 was found to be not different between paired fresh frozen and FFPE tissue samples. Thus, methylation levels of CpG site 3 or CpG sites 2 and 3 which were assayed in FFPE tissue samples were considered representative values of L1 methylation level.

For a total of 427 cases of CRC, methylation levels of L1 CpG

sites 2 and 3 were distributed with a mean value of 52.77%, median value of 51.68%, SD of 8.46%, range of 29.81 to 78.73, and interquartile range of 47.29 – 57.27. And methylation levels of L1 CpG site 3 were distributed with mean value of 54.96%, median value of 54.16%, SD of 9.70%, range of 23.74 to 86.10%, and interquartile range of 48.62 to 60.30%. According to methylation levels of L1, CRCs were grouped into quartile groups (Q1, Q2, Q3, and Q4). Methylation levels of L1 CpG sites 2 and 3 were divided into quartiles [Q1 (29.81%–47.24%, n=106), Q2 (47.29%–51.62%, n=107), Q3 (51.68%–57.21%, n=108), and Q4 (57.27%–78.73%, n=106)] and methylation levels of L1 CpG site 3 were divided into quartiles [Q1 (23.74%–48.58%, n=105), Q2 (48.62%–54.12%, n=108), Q3 (54.16%–60.28,% n=107), and Q4 (60.30%–86.10%, n=107)].

Relationships between L1 methylation level and clinicopathological features

When we analyzed associations between methylation level of L1 and clinicopathological parameters, methylation level of CpG

sites 2 and 3 or 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. Low methylation status of L1 was closely associated with nodal metastasis but methylation level of L1 was not different between N1 and N2 stage. Methylation level of L1 was significantly higher in CIMP-H, MSI-L/MSS tumors than in the other three molecular subtypes.(Table 1)

Table 1. Methylation level of L1 and clinicopathological features

Characteristic	No. of cases	L1		
		Mean	SD	P-value
Gender				
M	263	52.46	8.69	0.088
F	164	53.28	8.07	
Age				
≤59 years	209	53.09	8.19	0.344
≥60 years	218	52.47	8.72	
Site				
Right colon	139	52.85	8.81	0.628
Left colon	261	53.09	8.51	
Rectum	27	52.2	5.8	
T category				0.58
1	22	53.26	7.55	
2	371	52.54	8.37	
3	17	56	9.89	
4	17	53.97	9.92	
N category				**0.048**
0	63	55.05	9.54	
1	252	52.13	8.52	
2	112	52.94	7.47	
T histology				0.61
1	408	52.69	8.31	
2	19	54.68	11.3	
TNM staging				**0.02**
2	63	55.05	9.54	
3	364	52.38	8.21	
Angiolymphatic invasion				
present	180	53.3	8.7	0.409
absent	247	52.4	8.28	
Venous invasion				
present	42	53.4	8.57	0.803
absent	385	52.7	8.46	
Perineural invasion				
present	96	53.9	8.64	0.074
absent	331	52.5	8.39	
MSI				
MSI-(MSS/MSI-L)	396	53.04	8.39	0.34
MSI-H	40	51	8.68	
BRAF				
WT	399	52.96	8.45	0.241
Mutant	12	54.8	6.1	
KRAS				
WT	285	52.59	8.4	0.256
Mutant	109	53.75	8.52	

Survival

In the Kaplan–Meier survival analysis with log rank test, quartile groups of L1 CpG site 3 did not show statistical significance although Q4 group tended to be associated with higher DFS. 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. 1). In a univariate Cox regression analysis, compared to Q4 cases, Q1 cases experienced a significantly lower DFS rate (P = 0.017, HR 3.036, 95 % CI 1.224–7.439). The Q2 and Q3 cases experienced a slightly, but not significantly, lower DFS rate (Table 2). For the subsequent multivariate analysis, Q2, Q3, and Q4 were combined and defined as hypermethylated group, while Q1 was defined as hypomethylated group. Univariate analysis revealed that besides low methylation status of L1 CpG sites 2 and 3, T4 stage, N2 stage, lymphatic emboli, venous invasion, perineural invasion, mucinous histology, and KRAS mutation were associated with shorter DFS time. These parameters and low methylation status of L1 CpG sites 2 and 3 were incorporated into multivariate Cox

model, which revealed that L1 hypomethylation correlated independently with shorter DFS time (HR 1.858, 95 % CI 1.026–3.367, p = 0.041) (Table 3).

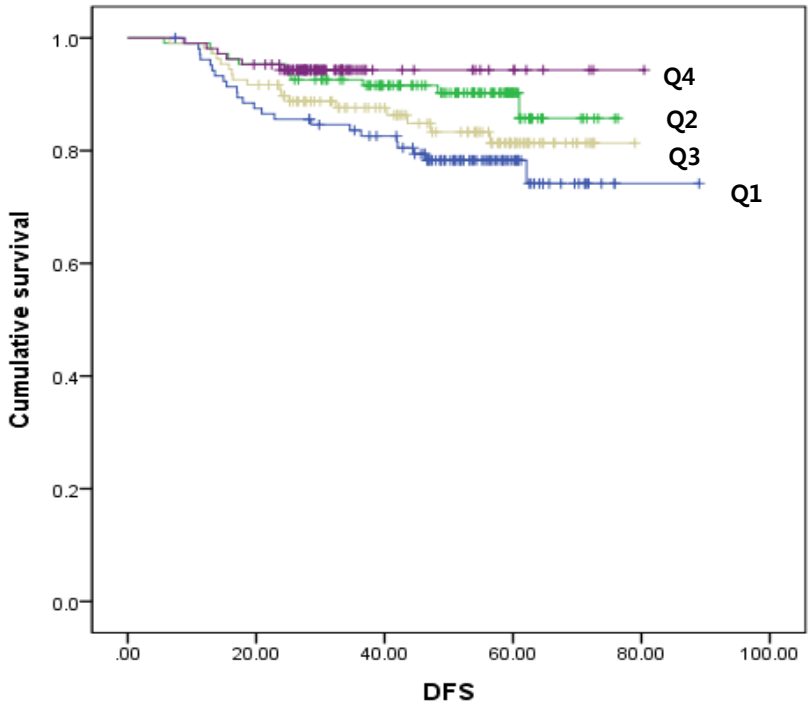

Figure 1. Kaplan-Meier survival analysis of quartile groups of LINE-1 CpG sites exhibited a significant difference in DFS.

Q1 group showed the lowest DFS, whereas Q4 group displayed the highest DFS

Table 2. In a univariate Cox regression analysis, compared to Q4 cases, Q1 cases experienced a significantly lower DFS rate. The Q2 and Q3 cases experienced a slightly, but not significantly, lower DFS rate.

	Disease-free survival		
Variable		HR (95% CI)	P-value
L1 methylation	Q4		
	Q1	3.036 (1.224-7.532)	0.017
	Q2	1.351 (0.496-3.684)	0.556
	Q3	2.228 (0.872-5.691)	0.094

Table 3. Univariate and multivariate survival analysis.

Characteristic	No. of cases	Univariate analysis DFS (mo) (95 % CI)	P-value	Multivariate analysis HR (95 % CI)	P-value
Gender					
M	301	71 (69-74)	0.57		
F	196	78 (75-82)			
Age					
≤59 years	243	75 (72-79)	0.99		
≥60 years	254	77 (74-80)			
Location					
Right colon	169	77 (73-80)	0.517		
Left colon	296	71 (68-73)			
Rectum	32	75 (65-84)			
T stage					
T1-3	474	78 (76-81)	0.002		
T4	23	55 (43-67)			
N stage					
N0-1	364	75 (73-77)	<0.001		
N2	133	68 (62-73)		1.917 (1.033-3.557)	0.039
T histology (mucin)					
low	472	78 (76-81)	0.006		
high	25	56 (45-67)			
TNM stage					
Ⅱ	74	73 (68-78)	0.52		
Ⅲ	423	77 (75-80)			
Angiolymphatic invasion					
present	180	71 (66-75)	<0.001	3.090 (1.609-5.935)	0.001
absent	247	80 (78-82)			
Venous invasion					
present	42	66 (57-75)	<0.001		
absent	385	77 (74-79)			
Perineural invasion					
present	96	61 (56-66)	<0.001		
absent	331	80 (77-82)			
KRAS					
WT	324	80 (78-83)	<0.001		
mutant	119	65 (30-70)		3.191 (1.822-5.588)	<0.001
BRAF					
WT	440	78 (75-80)	0.874		
mutant	16	64 (54-74)			
MSI					
MSS/MSI-L	464	77 (75-80)	0.455		
MSI-H	31	70 (63-76)			
L1					
Q1	105	74 (68-79)	0.028		
Q2	108	70 (67-74)			
Q3	108	69 (65-74)			
Q4	106	77 (74-80)			
L1					
Q1 (hypomethylated)	105	74 (68-79)	0.013	1.858 (1.026-3.367)	0.041
Q234 (hypermethylated)	322	73 (71-76)			

Discussion

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 the response to treatment and the clinical outcome. However, the quality of FFPE tissues depends on ischemic time before fixation, duration of fixation, and storage conditions which vary greatly from one sample to another. 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.[32-34] A recent study has raised a concern over the use of FFPE tissue for the assessment of DNA methylation by demonstrating a significant difference of L1 methylation level in 28% of paired fresh-frozen and FFPE tissue samples.[35] In the present study, of the four CpG sites which are targeted by most studies using pyrosequencing methylation assay, CpG site 3 was found to be the same in the methylation level between paired fresh-frozen and FFPE tissue samples. And of the two or more serial CpGs,

CpG sites 2 and 3 were the same in the methylation level between paired fresh-frozen and FFPE tissue samples, while the average level of CpG sites 1 to 3 or 4 were significantly different. A large-scaled study is required to evaluate whether CpG site 3 or CpG sites 2 and 3 are the same in methylation level between paired fresh-frozen and FFPE tissue samples regardless of duration of fixation or ischemic time.

In the present study, we found that tumoral L1 hypomethylation is 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.[32,33] However, these studies did not take into account the use of adjuvant chemotherapy in their analysis and thus did not consider its influence on the observed prognostic value of tumoral L1 hypomethylation. A recent study analyzed 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, showed that tumoral L1 hypomethylation is a marker of poor prognosis in

CRC patients who received surgery but no adjuvant chemotherapy.[36] This study has demonstrated no difference of survival between adjuvant-treated cancer patients with and without tumoral L1 hypomethylation, which is contrasted with findings of our study. The discrepancy might be related to difference in the methodology of 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, difference in the adjuvant chemotherapy is more likely to explain the discrepancy and it can be speculated that tumoral L1 hypomethylation might be 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 since 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.[37-39] However, prognostic potential of tumoral L1 hypomethylation has not been assessed in the adjuvant FOLFOX-treated

patients with resectable CRC. For the first time, our study has exhibited correlation between tumoral L1 hypomethylation and shorter DFS time in a cohort of patients with resectable CRC 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 CRC treated with adjuvant FOLFOX.In the present study, besides mucinous histology and KRAS mutation, L1 hypomethylation correlated independently with worse clinical outcome.

In the present study, tumor L1 hypomethylation has been demonstrated to be associated with shortened DFS time in patients with stage III or high-risk stage II CRC who were treated with adjuvant FOLFOX. The mechanism how low L1 methylation status contributes to tumor aggressiveness remains to be clarified. However, considering the fact that tumoral L1 hypomethylation is closely associated with chromosomal instability, it can be speculated that chromosomal instability represented by L1 hypomethylation might contribute to tumor aggressiveness. Furthermore, L1 hypomethylation dysregulate expression of genes which harbor L1 sequences in their

intronic or promoter sequences. Down-regulated tumor suppressor genes or over-expressed proto-oncogenes might contribute to tumor-aggressiveness. Microsatellite unstable CRCs are known to behave less aggressively in clinical outcome and also to be less hypomethylated in L1 sequences. Mutual exclusive relationship between microsatellite unstable CRCs and tumoral L1 hypomethylation might contribute to worse clinical outcomes of CRCs with low L1 methylation status. Findings of the present study has suggested that tumoral L1 hypomethylation is closely associated with shortened DFS time in adjuvant FOLFOX-treated patients with stage III or high-risk stage II CRC.

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국문초록

배경: 종양세포에서 Long intersperse element-1 (L1)의 저메틸화는 세포내 메틸싸이토신 함량 감소를 대표하는 표지자로 인식되어지며, 위암과 식도암의 환자에서 종양의 L1 저메틸화는 나쁜 예후와 관련되어 있다. 하지만, L1 메틸레이션의 저발현이 결,직장암의 예후인자인지에 대해서 아직 증명되어 있지 않다. 본 연구는 FOLFOX 치료를 받은 결,직장암 환자에서 종양의 L1 저메틸화가 예후와 관련되어 있는지를 밝히는 것을 연구목표로 하였다.

연구대상 및 방법: 본 연구에서는 427개의 수술에서 절제한 stage Ⅲ 혹은 high-risk stage Ⅱ 단계의 결,직장암 환자 샘플을 수집하고 이런 샘플로 L1 메틸레이션, CpG island 메틸레이션 표현형, microsatellite 의 불안정성 그리고 KRAS/BRAF 돌연변이 분석을 시행하였다. 그 중 L1 메틸레이션은 pyrosequencing으로 분석하였다.

결과: L1의 저메틸화는 림프절에의 전이와는 밀접한 관계가 있다고 알려져 있지만 환자의 나이, 성별 그리고 종양의 표현형 및

분화, 점액소의 조직학구조, 림프절의 색전, 혈관과 신경의 침식, T stage, KRAS/BRAF의 돌연변이 등과는 관련이 있다고 보고되어 있지 않다. 본 연구는 여러가지 실험을 거쳐 L1의 저메틸화는 점액성 선암종, T stage, N stage, 림프절의 색전, KRAS 돌연변이와 같이 나쁜 예후를 예측할 수 있는 독립적인 예후인자라는 것을 보여주고 있다.

결론: 대장암 결제수술을 받고 FOLFOX 치료를 받은 환자에서 종양의 L1 저메틸화는 불량한 예후와 관련된 독립적인 나쁜 예후 인자이다.

주요어: LINE-1, 결,직장암, FOLFOX, 예후인자, 저메틸화

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