이학석사 학위논문

Tumoral LINE-1 hypomethylation is associated with poor survival of patients with intrahepatic cholangiocarcinoma

LINE-1의 저메틸화와
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Abstract

DNA methylation changes occurring in cancer cells are characterized by both promoter CpG island hypermethylation and diffuse genomic hypomethylation. LINE-1 is half-million times repeated in the human genome in an interspersed manner and CpG sites located in 5' untranslated region of LINE-1 are heavily methylated in normal cells and undergo demethylation in association with cancerization. However, little information is available regarding LINE-1 hypomethylation and its prognostic implication in intrahepatic cholangiocarcinomas. We analyzed 172 cases of intrahepatic cholangiocarcinomas for their methylation status at four CpG sites of LINE-1 using pyrosequencing and correlated LINE-1 methylation level with clinicopathological features. Tumor differentiation, lymphatic invasion, and T stage were associated with a low average methylation level of LINE-1 at the four CpG sites; LINE-1 methylation level tended to be lower in high grade differentiation, lymphatic emboli, and higher T stage. Lower methylation status of LINE-1 was significantly associated with lower overall survival in patients with intrahepatic cholangiocarcinoma and found to be an independent prognostic parameter. Our findings suggest that tumoral LINE-1 hypomethylation could be a molecular biomarker heralding poor prognosis of patients with intrahepatic cholangiocarcinoma. A further study is required to validate our findings.

Key Words: Cholangiocarcinoma, LINE-1, methylation, prognosis, pyrosequencing

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Introduction

DNA methylation changes occurring in cancer cells are characterized by focal promoter CpG island hypermethylation and diffuse genomic hypomethylation. Promoter CpG island hypermethylation contributes to inactivation of tumor suppressor genes or tumor-related genes, whereas diffuse genomic hypomethylation is associated with chromosomal instability (1). Repetitive DNA elements comprise approximately half of the human genome and Long interspersed element-1 (LINE-1) retrotransposons comprises approximately 18% of the human genome (2). 5' untranslated region sequence of LINE-1 has a high density of CpG dinucleotides which are heavily methylated in normal cells but undergoes hypomethylation in most tissue types of human cancer (3). Since Weisenberger et al' study demonstrated a close correlation between genomic DNA methylation levels, determined by high-performance liquid chromatography, and LINE-1 DNA methylation levels determined by PCR-based measurement (4), LINE-1 methylation levels assessed by PCR-based methylation assays have been considered a surrogate marker for genomic methylation levels.

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignancy which arises from any portion of the intrahepatic biliary tree. ICC is a fatal disease because of detection at a late stage in its course, frequent lymphovascular or perineural invasion, and lack of effective therapeutic modalities (5, 6). Cancer staging and subsequent allocation to the optimal treatment approach is crucial for ICCs. However, none of the existing staging system,

including the 7th version of the American Joint Cancer Committee/Union for International Cancer Control (AJCC/UICC) staging system, fulfills the criteria of optimal staging system(7). The current version of AJCC/UICC tumor, lymph node, metastasis (TNM) staging system for ICCs has been controversial for its prediction power of prognosis (8, 9) because a recent study of the Japanese Liver Cancer Study Group has demonstrated no difference in overall survival between TNM stage II and III ICCs (9). Although more works should be done for the optimization of the existing staging systems, molecular biomarkers associated with clinical outcome can help to predict tumor behavior and clinical outcome and need to be developed.

Hypomethylation of LINE-1 in tumors has been associated with poor prognosis of patients in many tissue types of human cancer, including gastric cancer and colon cancer (10-15). And furthermore, an independent association of tumoral LINE-1 hypomethylation with poor prognosis of cancer patients has been demonstrated in the colon, stomach, esophagus, liver, lung, and brain (12, 15-18). In the literature, however, no information is available regarding prognostic implication of LINE-1 methylation status in ICCs. In the present study, we analyzed ICCs for their methylation levels of LINE-1 using pyrosequencing methylation assay and correlated LINE-1 methylation status with clinicopathological features including survival.

Material and methods

A total of 172 formalin-fixed, paraffin-embedded archival tissue samples were obtained from patients who underwent surgical resection for ICC at the Seoul National University Hospital, Seoul, South Korea, from April 2005 to December 2012. fifteen non-neoplastic gallbladder tissue samples were obtained from patients with chronic cholecystitis. Hilar cholangiocarcinomas which arise from the left and right hepatic ducts at or near their junctions were excluded from the study. Through meticulous histological examinations, combined hepatocellular carcinoma and cholangiocarcinoma were excluded from the study. This retrospective study was approved by the Institutional Review Board at the Seoul National University Hospital (IRB No. H-1011-046-339). All cases were reviewed by experienced gastrointestinal pathologists (KBL and JJJ) to confirm the diagnosis of ICC and to re-evaluate histological findings and TNM stages according to the 4th edition 2010 WHO classification and the 7th edition 2009 AJCC/UICC staging system, respectively (19, 20). Gross types of ICC were classified as are classified into three types according to gross appearance, including mass-forming (MF) type, periductal infiltrative (PI) type, and intraductal growth (IG) type (21, 22). For multiple tumor ICCs, gross pathological classification was performed according to larger tumor.

DNA extraction and bisulfite modification

Through microscopic examination, tumor areas where the tumor cells comprised >50% of total neoplastic and non-neoplastic cells and

represented the most prevalent histological type of the individual case were marked with a marker pen. For cases with ICC of multiple-tumor type, tumor areas were marked in the larger or the largest tumor of the case. The corresponding areas were scraped from unstained tissue glass slides with a knife blade. For non-neoplastic gallbladder tissue samples (n=15), mucosal tissues were scraped from the unstained tissue glass slides. The scraped tissue was collected into microtubes containing 50 μl of tissue lysis buffer and proteinase K. After incubation of the tubes for 48h at 55℃, the lysates were subjected to heating at 95℃ for 30 min for inactivation of proteinase K, which was found to be necessary for lessening formalin fixation-induced discrepancy in the measured value of LINE-1 methylation level (submitted). Following centrifugation of the tissue lysates, the supernatants were transferred into new tubes. Bisulfite modification of DNA samples was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA).

LINE-1 PCR and Pyrosequencing

LINE-1 methylation levels were assessed using a pyrosequencing methylation assay. The primers and PCR conditions were described previously (11). The LINE-1 assays were performed in a 25-μl PCR reaction containing 2-μl bisulfite-treated genomic DNA, 60 mM Tris-HCl (pH 8.8), 15 mM ammonium sulfate, 0.5 mM $MgCl_2$, 1mM dNTP mix, and 1U of Taq polymerase. The PCR products were purified and quantified in the PyroMarkQ24 System (Qiagen, Valencia, CA, USA). The amounts of C nucleotides relative to the sum of C and T nucleotides at each CpG site were calculated as percentages. The average of four percentage values in the four adjacent CpG sites (nucleotide positions 328, 321, 318, and 306 of X58075 (GenBank)) was taken as the overall

LINE-1 methylation level in a given sample.

Statistical analysis

Because LINE-1 methylation data followed a normal distribution, we used parametric tests that compare groups. However, when the following criteria were not met, we used both parametric tests and non-parametric tests: when two or more groups were compared, each group should be greater than 15. Parametric tests (student t-test and ANOVA test) were performed for comparison of two groups and three or more groups, respectively. Non-parametric tests (Mann-Whitney test and Kruskal-Wallis test) were further performed for the comparison of two groups and three or more groups, respectively when one group was not greater than 15. Overall survival was defined as the time from surgery to death from any cause. Kaplan-Meier log rank test and Cox-proportional hazard method were used for survival analysis. For multivariate analysis, variables which were found to be significant in univariate analysis were included in Cox-proportional hazard model, then statistically significant variables were selected by backward elimination. All p values were two-sided, and the statistical significance was set at $p < 0.05$. SPSS software (IBM SPSS Statistics version 23; Chicago, IL, USA) was used for all statistical analyses.

Results

Demographic and clinicopathological data

One hundred seventy-two ICC patients underwent hepatic resection between 2005 and 2012. Of the patients, 147 patients (85.5%) presented with a single tumor. Male to female ratio was 121:51 and average age was 62.7 years (median, 63 years; range, 38-80 years). Gross type was MF type in 141 patients, PI type in 8 patients, IG type in 18 patients, and MF plus PI type in 5 patients. Stage grouping was stage I in 40, stage II in 37, stage III in 30, and stage IV in 65. Grading was well differentiated in 23, moderately differentiated in 94, and poorly differentiated in 55. Demographic and clinicopathological findings are summarized in Table 1.

Relationship between LINE-1 methylation level and clinicopathological features

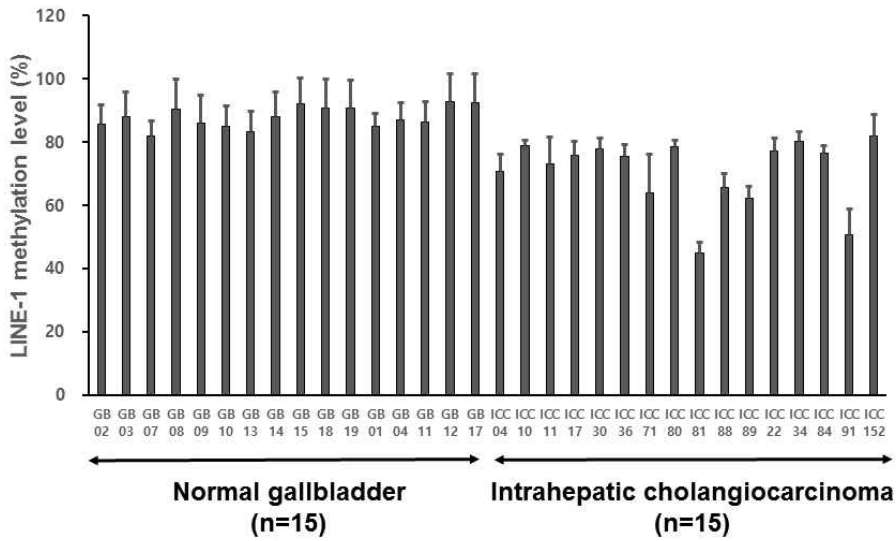
LINE-1 methylation level was significantly lower in ICC tissue samples than in normal gallbladder tissue samples (Fig. 1). Tumoral LINE-1 methylation levels were not different in ICCs between male and female patients and between younger and older patients (<63 years old and ≥ 63 years). No association was found between tumoral LINE-1 methylation levels and gross types. Tumoral LINE-1 methylation levels were not different between ICCs of single-tumor and multiple-tumor types. However, a significant difference was noted between well-differentiated ICCs and moderately or poorly-differentiated ICCs: well-differentiated ICCs showed higher methylation levels than those of

moderately or poorly-differentiated ICCs. Tumoral LINE-1 methylation levels tended to be higher in T1 stage than in T2b or higher T stages. No difference of LINE-1 methylation levels was found between ICCs of N0 and N1, or between ICCs of M0 and M1. While LINE-1 methylation levels were significantly lower in ICCs with lymphatic tumor emboli than in ICCs without lymphatic tumor emboli, no significant difference was noted between ICCs with and without venous tumor emboli and between ICCs with and without perineural invasion.

Table 1. Relationship between LINE-1 methylation level and clinicopathological parameters

		No.	Average	SD	P-value
Sex	M	121	75.48	7.173	0.233
	F	51	76.97	8.216	
Age	<64 years	87	75.97	8.274	0.936
	≥64 years	85	75.87	6.674	
Gross type	Mass forming	141	75.66	7.616	0.702 (ANOVA)
	Periductal	8	78.62	3.790	0.558 (KW)
	Intraductal	18	76.73	7.110	
	Mixed	5	76.16	10.837	
Multiplicity	single	147	75.73	7.898	0.422
	multiple	25	77.04	4.501	
T stage	pT1	48	78.08	6.972	
	pT2a	38	73.08	9.417	
	pT2b	14	76.12	4.010	0.049
	pT3	47	76.04	7.026	0.012 (MW)
	pT4	25	75.74	6.622	
N stage	pN0	86	74.87	8.214	0.536
	pN1	39	75.78	6.223	
M stage	pM0	106	75.15	8.264	0.259
	pM1	11	72.26	4.972	0.035 (MW)
TNM stage	I	40	77.81	7.579	0.229
	II	37	74.42	8.747	
	III	30	76.25	7.801	
	IV	65	75.45	6.406	
Differentiation	Well	23	79.69	4.766	0.014
	Moderate	94	75.95	6.857	
	Poorly	55	74.28	8.906	
Neural invasion	Absent	118	75.77	8.146	0.701
	Present	54	76.25	5.921	
Lymphatic invasion	Absent	102	76.92	7.200	0.034
	Present	70	74.46	7.749	
Vascular invasion	Absent	91	76.26	8.176	0.396
	Present	77	75.27	6.631	
Chronic liver disease	Absent	130	75.95	7.497	0.936
	Present	42	75.84	7.617	
Chronic biliary disease	Absent	159	75.93	7.679	0.962
	Present	13	75.82	5.108	

Figure 1. LINE-1 methylation level was significantly lower in tumor tissues of intrahepatic cholangiocarcinoma (n=15) than in mucosal tissues of non-neoplastic gallbladder (n=15) (student t-test, p-value <0.001).



Relationship between tumoral LINE-1 methylation status and overall survival of patients with ICC

When ICCs were grouped into four quadrants (Q1, Q2, Q3, and Q4 in the order of increasing level of LINE-1 methylation) according to their tumoral LINE-1 methylation levels, Q1 and Q2 showed significantly lower overall survival than that of Q3 and Q4 (Fig. 2). As displayed in the Kaplan-Meier survival curve, overall survival curves of Q1 and Q2 are similar, while overall survival curves of Q3 and Q4 are similar. Thus, ICCs were further grouped into low methylation status subgroup (Q1 and Q2) and high methylation subgroup (Q3 and Q4). Low LINE-1 methylation status was associated with worse overall survival in Kaplan-Meier survival analysis. Besides low LINE-1 methylation status, T staging, N staging, lymphatic emboli, perineural invasion, and histological differentiation were included into a multivariate analysis which revealed that a low LINE-1 methylation status was independently associated with worse overall survival in patients with ICC (Table 2&3).

Figure 2. Kaplan-Meier survival analysis with log rank test. ICC patients were grouped into 4 quadrants according to their tumoral LINE-1 methylation levels. Q1, 2, 3, and 4 are in the order of increasing methylation level of LINE-1. (A) Overall survival was lower in patients with Q1 or Q2 ICC than in patients with Q3 or Q4 ICC. (B) When ICC patients were grouped into low LINE-1 methylation status (Q1 and 2) and high LINE-1 methylation status (Q3 and 4), overall survival was significantly lower in low methylation status group than in high methylation status group.

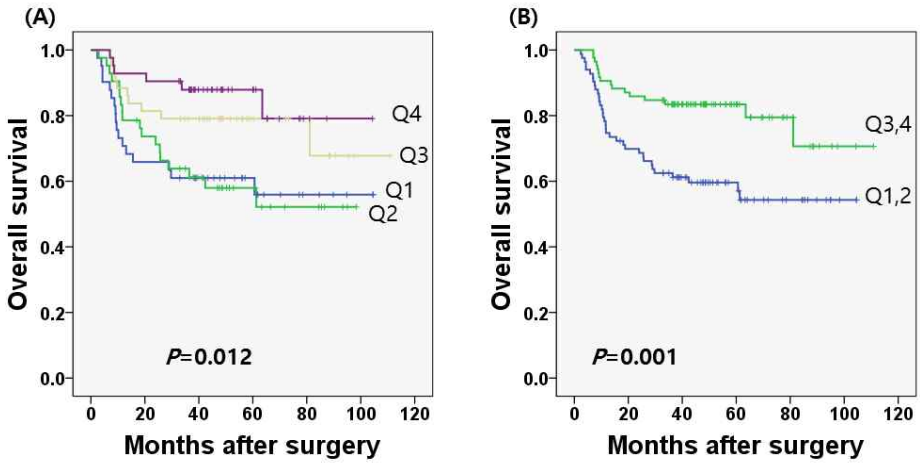


Table 2. Univariate survival analysis of LINE-1 methylation level and clinicopathological parameters with respect to overall survival

Parameters		Hazard ratio (95% C.I.)	P-value
LINE-1 methylation status			0.019
	Q4 (n=42)	Reference	
	Q3 (n=42)	1.672 (0.807-4.602)	0.320
	Q2 (n=42)	3.361 (1.333-8.477)	0.010
	Q1 (n=42)	3.462 (1.364-8.789)	0.009
LINE-1 methylation status	Q3, Q4 (n=84)		
	Q1, Q2 (n=84)	2.552 (1.412-4.614)	0.002
pTNM staging			0.792
	I (n=40)	Reference	
	II (n=37)	1.245 (0.549-2.825)	0.600
	III (n=30)	0.925 (0.358-2.395)	0.873
	IV (n=65)	1.332 (0.637-2.789)	0.446
T staging			0.055
	pT1 (n=47)	Reference	
	pT2a (n=37)	1.711 (0.831-3.523)	0.145
	pT2b (n=14)	2.155 (0.864-5.376)	0.100
	pT3 (n=45)	0.913 (0.413-2.019)	0.821
	pT4 (n=25)	0.400 (0.115-1.398)	0.151
N staging	pN0 (n=85)		
	pN1 (n=39)	2.211 (1.176-4.157)	0.014
M staging	pM0 (n=158)		
	pM1 (n=10)	1.649 (0.593-4.583)	0.337
Gross type			0.104
	Mass forming (n=138)	Reference	
	Periductal (n=17)	0.272 (0.066-1.124)	0.072
	Intraductal growth (n=8)	0	0.976
	Mixed (n=5)	2.654 (0.821-8.586)	0.103
Lymphatic emboli	Absent (n=101)		
	Present (n=67)	2.526 (1.444-4.417)	0.001
Vascular invasion	Absent (n=89)		
	Present (n=76)	1.077 (0.614-1.889)	0.796
Perineural invasion	Absent (n=115)		
	Present (n=53)	0.378 (0.178-0.805)	0.012
Tumor border	Expanding (n=32)		
	Infiltrative (n=136)	1.846 (0.787-4.330)	0.159

Tumor differentiation			0.099
	Well (n=22)	Reference	
	Moderate (n=93)	4.511 (1.079-18.867)	0.039
	Poor (n=53)	4.929 (1.141-21.283)	0.033

Table 3. Multivariate survival analysis of LINE-1 methylation level and clinicopathological parameters with respect to overall survival.

		Hazard ratio (95% C.I.)	P-value	Hazard ratio (95% C.I.)	P-value
LINE-1 methylation level	Q3, Q4 (n=84)				
	Q1, Q2 (n=84)	2.552 (1.412-4.614)	0.002	2.360 (1.129-4.934)	0.022
N staging	pN0 (n=85)				
	pN1 (n=39)	2.211 (1.176-4.157)	0.014	2.241 (1.130-4.444)	0.021
T staging			0.031		0.136
	pT1 (n=47)				
	pT2 (n=51)	1.824 (0.933-3.567)	0.079	0.456 (0.144-1.440)	0.181
	pT3 (n=45)	0.913 (0.413-2.018)	0.821	0.342 (0.096-1.219)	0.098
	pT4 (n=25)	0.400 (0.115-1.397)	0.151	0.174 (0.039-0.794)	0.023
Lymphatic emboli	Absent (n=101)				
	Present (n=67)	2.526 (1.444-4.417)	0.001	3.624 (1.335-9.835)	0.011
Perineural invasion	Absent (n=115)				
	Present (n=53)	0.378 (0.178-0.805)	0.012	0.433 (0.183-1.023)	0.056
Differentiation			0.099		0.458
	Well (n=22)				
	Moderate (n=93)	4.511 (1.079-18.867)	0.039		
	Poor (n=53)	4.929 (1.141-21.283)	0.033		

Discussion

Relationships between low tumoral LINE-1 methylation status and poor survival have been demonstrated in several types of gastrointestinal tract malignancy, including esophageal squamous cell carcinoma, gastric adenocarcinoma, and colorectal adenocarcinoma. However, no study is available regarding the relationship between tumoral LINE-1 hypomethylation and survival of patients with ICC. In the present study, we have for the first time demonstrated a close association between low tumoral LINE-1 methylation status and poor survival of patients with ICC. In our previous study, levels of LINE-1 methylation have been demonstrated to be lower in extrahepatic cholangiocarcinomas (ECCs) than in normal bile ducts and the timing of tumoral LINE-1 hypomethylation is a late event during multistep carcinogenesis of ECC (23). However, our previous study did not analyze the relationship between tumoral LINE-1 methylation status and survival of patients with ECC. In the present study, tumoral LINE-1 hypomethylation was found to be an independent parameter heralding poor prognosis in patients with ICC.

Many tissue types of human cancer have demonstrated close associations between tumoral LINE-1 hypomethylation and poor prognosis of patients with the specific tissue type of cancer. However, no satisfactory explanation has been provided regarding the reason why tumoral LINE-1 hypomethylation status contributes to the aggressive behavior of the tumor, which is the same case to ICCs. Several speculations might be made regarding the mechanism how tumoral

LINE-1 hypomethylation contributes to poor survival in the patients with ICC. Decreased methylation level of LINE-1 might lead to increased genomic instability through enhanced non-homologous recombination and subsequent chromosomal instability, increased retrotransposon activity and subsequent random insertional mutation, or decreased mRNA expression of genes harboring anti-directional LINE-1s in their intron sequences. Such an enhanced genomic instability might cause an increased expression of proto-oncogenes or a decreased expression of tumor-suppression genes, which might contribute to increased aggressiveness of ICCs. However, the exact mechanism how tumoral LINE-1 hypomethylation contributes to the aggressive behavior is unknown.

The tumor, lymph node, metastasis (TNM) system is used to stage ICCs and staging helps to decide on the right treatment. However, the prognostication power of the current version TNM staging system for ICCs has been dubious because in a recent study, overall survival was not different between stage II and III ICCs (9). Because of weak discriminating power of the current TNM staging system, it is necessary to develop molecular markers which are able to predict tumor behavior and will help to predict the risk of recurrence after the surgery and to plan more effective cancer treatments. In a recent next generation sequencing-based study, a clustering analysis of global gene expression levels has been shown to predict prognosis of patients with biliary tract cancer (24). ICCs belonging to a cluster with high frequency of mutations in *BAP1*, *IDH1*, or *NRAS* tended to exhibit better clinical outcome compared with ICCs belonging to two clusters with high frequency of mutations in *TP53*, *KRAS*, or *SMAD4*. However, the clustering analysis of global gene expression levels should be validated

in an independent study for its usefulness for prediction of prognosis. In the literature, however, there are no single molecular markers, except for immunohistochemical markers, which have been demonstrated to be closely associated with clinical outcome of patients with ICC. Our findings indicated that tumoral LINE-1 hypomethylation status was closely associated with poor prognosis in patients with ICC and that tumoral LINE-1 hypomethylation was an independent biomarker heralding poor prognosis in patients with ICC. However, this finding should be validated in an independent set of ICCs.

In the present study, because of the association between tumoral LINE-1 methylation level and lymphatic emboli both of which were independent prognostic parameters in ICCs, we expected to develop combinatory markers which are superior to each alone in the aspect of prognostication power. For this aim, ICCs were divided into two groups (ICCs with lymphatic emboli vs. without lymphatic emboli) according to lymphatic emboli. Then, univariate analysis was performed in patients of ICCs to elucidate whether combination of both parameters, LINE-1 methylation and lymphatic embolus statuses, contribute to identification of a subgroup of ICCs with poor prognosis. Compared with ICCs with high tumoral LINE-1 methylation status and no lymphatic tumor emboli, ICCs with low tumoral LINE-1 methylation status and lymphatic tumor emboli showed hazard ratio of 3.609 (1.639-7.945) whereas ICCs with low tumoral LINE-1 methylation status and no lymphatic tumor emboli harbored hazard ratio of 0.858 (0.303-2.430) (Table 4).

In summary, we have analyzed 172 cases of ICC for their methylation status in LINE-1 using pyrosequencing and correlated LINE-1

methylation status with clinicopathological features of ICCs. We found that tumoral LINE-1 hypomethylation was an independent prognostic parameter heralding poor prognosis of ICC patients. Further study is required to validate our findings of tumoral LINE-1 hypomethylation as a prognostic marker.

Table 4. Multivariate survival analysis of combinatory LINE-1 methylation level and lymphatic embolus statuses and clinicopathological parameters with respect to overall survival.

Parameters		Hazard ratio (95% C.I.)	P-value
LINE-1 methylation /lymphatic emboli status			<0.001
	High/absent (n=37)	Reference	
	Low/present (n=33)	3.609 (1.639-7.945)	0.001
	Low/absent (n=35)	0.858 (0.303-2.430)	0.774
	High/present (n=19)	0.506 (0.104-2.453)	0.397
T staging			0.244
	pT1 (n=47)	Reference	
	pT2 (n=51)	0.502 (0. 157-1.598)	0.243
	pT3 (n=45)	0388 (0.108-1.393)	0.147
	pT4 (n=25)	0.219 (0.048-1.000)	0.050
N staging	pN0 (n=85)		
	pN1 (n=39)	2.322 (1.043-5.167)	0.029
Perineural invasion	Present (n=53)		
	Absent (n=115)	2.096 (1.081-4.067)	0.039
Differentiation			0.305
	Well (n=22)	Reference	
	Moderate (n=93)	3.116 (0.857-14.784)	0.152
	Poor (n=53)	3.403(0.713-16.248)	0.125

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국 문 초 록

암세포에서 나타나는 DNA 메틸화(Methylation)의 변화양상으로는 프로모터에 있는 CpG island에서의 과메틸화와 퍼져있는 CpG site에서의 저메틸화가 있습니다. LINE-1은 사람의 DNA 사이 사이에 끼어 들어가는 방식으로 50만번 반복되어 있고, LINE-1의 5‘ 비번역부위에 있는 CpG site들은 정상 세포에서 많이 메틸화되어 있다가 암세포화 되면서 탈메틸화 됩니다. 그러나 LINE-1 저메틸화와 간내담관암(intrahepatic cholangiocarcinoma; ICC)의 예후예측에 관해서는 정보가 많지 않습니다. 우리는 파이로시퀀싱을 사용하여 172개의 간내담관암 샘플에서 4군데의 LINE-1 CpG site에서의 메틸화 상태를 분석하였고, LINE-1의 메틸화 정도와 임상병리학적양상과의 연관성을 확인하였습니다. 종양 분화, 림프관 침윤, T stage는 4개의 LINE-1 CpG site의 메틸화 평균값이 낮은 것과 관련이 있었습니다 ; LINE-1 메틸화 정도는 고도의 분화, 림프관 색전, 높은 T stage를 보이는 경우에서 낮아지는 경향을 보였습니다. LINE-1의 더 낮은 메틸화 상태는 간내담관암 환자의 낮은 전체생존율(Overall survival)과 관련이 있었고, 독립적 예후변수로써의 가능성을 확인하였습니다. 우리의 연구는 종양의 LINE-1 저메틸화가 간내담관암 환자의 안 좋은 예후를 알리는 분자적 생체표지자가 될 수 있다고 제안합니다. 이 발견을 입증하기 위해 후속연구가 필요합니다.

주요어 : 담낭 및 담관암, LINE-1, 메틸화, 예후, 파이로시퀀싱
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