Genome-Wide Hypomethylation and Specific Tumor-Related Gene Hypermethylation are Associated with Esophageal Squamous Cell Carcinoma Outcome

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Introduction: Esophageal squamous cell carcinoma (ESCC) is a cancer of variable outcomes with limited effective treatments resulting in poor overall survival (OS). Epigenetic alterations contributing to this deadly cancer type that can be used as novel therapeutic or diagnostic targets are still poorly understood.

Methods: We explored genome-wide DNA methylation data from The Cancer Genome Atlas project and identified a panel of tumor-related genes hypermethylated in ESCC. The methylation statuses of *RASSF1*, *RARB*, *CDKN2A (p16INK4a, p14ARF)*, *APC*, and *RUNX3* genes and long interspersed nucleotide element-1 (LINE-1) were validated in a large cohort (n = 140) of clinically well-annotated ESCC specimens and esophageal normal mucosa (n = 28) using a quantitative methylation-specific polymerase chain reaction.

Results: Hypermethylation of *RARB*, *p16INK4a*, *RASSF1*, *APC*, *RUNX3*, and *p14ARF* were observed in 55%, 24%, 20%, 19%, 14%, and 8% of specimens, respectively. Hypermethylation of *APC* was significantly associated with tumor depth (p = 0.02) and American Joint Committee on Cancer stage (p = 0.03). Global DNA methylation level, assessed by LINE-1, was significantly lower in ESCC than in normal mucosa ($p < 0.0001$), and lower in greater than or equal to T2 (n = 69) than T1 tumors (n = 45; p = 0.03). There was a significant inverse correlation between LINE-1 and *RARB* methylation (p = 0.008). Importantly, hypermethylation of *RASSF1* and *APC* genes was significantly associated with overall survival (OS; p = 0.006 and p = 0.007, respectively). In addition, patients with tumors containing a higher number of methylated genes (greater than two genes) presented worse OS (p = 0.003).

Conclusions: This study demonstrates that epigenetic alterations of a panel of tumor-related genes and the noncoding region LINE-1 can be used as prognostic indicators and help in clinical management of ESCC patients.

Key Words: Esophageal squamous cell carcinoma, LINE-1, DNA methylation, Prognosis, Tumor-related genes.

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Esophageal cancer can be differentiated into two histological subtypes: squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESCC) is a common malignancy worldwide, and its incidence is known to be high in African and Asian countries, particular in China, India, Japan, and other Southeast Asian countries.[1] In general, overall 3- and 5-year survival rates for ESCC patients who underwent esophagestomy have been reported to be 31.1–52.5% and 20.8–40.0%, respectively.[2,3] Although recent advances in surgical techniques and the introduction of preoperative chemoradiotherapy have been shown to significantly improve ESCC patients' survival, ~30% of patients still develop tumor recurrence by the time of 5-year follow-up.[4] Therefore, it is critical to improve risk assessment that can identify patients at high risk for recurrence. ESCC has defined clinicopathological prognostic factors such as lymph node metastasis, depth of tumor invasion, grade of differentiation, and family history; however, new prognostic factors are still needed to assess tumor progression and disease outcome.[2,5] Although some genomic aberrations have been recently identified as potential biomarkers for ESCC,[6,7] the clinical or pathological significance of these biomarkers is still not clear. Further studies to identify novel molecular alteration of ESCC are urgently needed.

ESCC etiology is often correlated with excessive consumption of carcinogenic substances such as alcohol and tobacco.[8,9] Tobacco exposure has been correlated with aberrant promoter hypermethylation of selected genes in esophageal noncancerous mucosae.[10] Although the mechanism of ESCC progression is poorly understood, it is suggested that epigenetic events may be involved.[11] Therefore, identifying aberrant epigenetic events is important to understand the

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ESCC progression and also to develop new potential biomarkers for ESCC. Genome-wide DNA hypomethylation and gene-specific hypermethylation are common epigenetic events in tumorigenesis for many types of carcinomas. Tumor-related gene (TRG) promoter CpG island hypermethylation is a well-recognized mechanism of transcriptional silencing.[12] Aberrant DNA methylation of TRG promoter regions has been recognized in different tumor cells.[13,14] Approximately 45% of the human genome is composed of transposable elements with interspersed repeats. Long interspersed nucleotide element-1 (LINE-1)[15] is the most abundant human non-long terminal repeat retrotransposable element, accounting for about 20% of the genome. Transcriptionally activated LINE-1 is related to hypomethylation of its promoter region. LINE-1 can be transposed, via reverse transcription, to different genomic regions causing disruption of genes and leading to genomic instability.[16] LINE-1 is hypermethylated in normal cells and has been shown to be hypomethylated in several types of carcinomas.[17–20] Our *hypothesis* is that epigenetic aberrations of key TRGs and LINE-1 are related to ESCC progression and disease outcome.

Using highly sensitive assays, absolute quantitative assessment of methylated alleles,[14] and methylation-specific PCR (MSP), we examined whether genome-wide hypomethylation and TRG-specific hypermethlyation contribute to ESCC progression and disease outcome. Initially, we explored TRGs in ESCC using genome-wide DNA methylation data from The Cancer Genome Atlas (TCGA) project. A panel of six CpG islands located in *RASSF1, RARB, p16INK4a, RUNX3, APC,* and *p14ARF* genes were significantly hypermethylated in ESCC. Several of these regions have been described to be hypermethylated[21–24] and correlated with disease outcome to some extent in ESCC.[25,26] *RASSF1*, *RARB,* and *RUNX3* gene hypermethylations have been shown to positively correlate with ESCC progression.[22–24,27] However, they have not been assessed in combination for ESCC primary tumor progression and overall disease outcome.

In this study, we identified a panel of epigenetic biomarkers for ESCC, including hypomethylation assessed by quantitative LINE-1 methylation and hypermethylation of six TRGs. This novel panel was shown to have significant prognostic utility in disease outcome in a multivariate analysis.

METHODS

Patients and Specimens

Representative blocks of paraffin-embedded archival tissue specimens were collected from 114 patients with ESCC that underwent surgery at Keio University Hospital in Tokyo, Japan from 1997 until 2002. The study design was approved by the institutional review board of Keio University. Since 1994, patients with suspected T4 tumors underwent chemoradiotherapy with 5-fluorouracil and cisplatin with a final objective of tumor irradiation. If downstaging was successful, patients underwent elective resection. In this cohort, none of the patients had prior chemoradiotherapy. Of the 114 ESCC patients, corresponding paired 26 available lymph node metastases were studied. In addition, from the primary tumor sections, adjacent normal esophageal epithelia (greater than 1 mm separated from cancer cells) were microdissected as normal control ($n = 28$, American Joint Committee on Cancer [AJCC] stage: I [$n = 12$], IIA [$n = 2$], IIB [$n = 10$], or III [$n = 4$], gender: male [$n = 25$], and female [$n = 3$], age: 44–75 [Mean = 60]). Patients were classified according to AJCC criteria.

DNA Isolation and Sodium Bisulfite Modification

Genomic DNA was extracted from paraffin-embedded archival tissue and the concentration was determined as we previously described.[28] An aliquot of 300 ng was subject to a sodium bisulfite modification reaction, as we previously described.[28]

Quantitative Analysis of Hypomethylated LINE-1

In a previous study, we introduced the absolute quantitative assessment of methylated alleles assay based on bisulfate-modified DNA and real-time polymerase chain reaction (PCR) using two *Taq*Man minor groove binder (MGB) probes labeled with different fluorophores.[14,29,30] In brief, a universal set of primers is utilized to amplify sodium bisulfite-modified LINE-1, despite its methylation status. These primers were designed as we previously described.[20] Then, a pair of MGB probes are utilized to specifically amplify methylated and unmethylated sequences for quantitative analysis. PCR amplification was performed with PCR buffers and forward/reverse primers, methylated- and unmethylated-specific MGB probes, dNTPs, 0.7 U of AccuStart *Taq* DNA polymerase (Quanta BioSciences, Gaithersburg, MD), and 1 μl bisulfite-treated DNA. Samples were amplified with a precycle hold at 95°C (10 min), followed by 45 cycles of denaturation at 95°C (15 sec), and annealing and extension at 60°C (1 min). The standard curve for quantifying methylated and unmethylated copy numbers was generated by a threshold cycle of serial dilutions of plasmid templates (10^6 to 10^1 copies). The mean of triplicate experiment was used as the copy number. LINE-1 hypomethylated controls (melanoma lines) and LINE-1 methylated controls (normal peripheral blood leukocytes) were included as assay controls in each 384-well-PCR microplates. The hypomethylated LINE-1 level was defined as the copy number of unmethylated LINE-1 relative to the sum of the copy number of methylated and unmethylated LINE-1 (U/U + M), referred as the LINE-1 U Index. The LINE-1 U index levels of each sample were compared as a representation of hypomethylated LINE-1 status.

Genome-Wide DNA Methylation Analysis of ESCC and Normal Esophagus

Genome-wide DNA methylation data for 38 ESCCs and 12 normal esophagus tissues (Supplementary Table 2, SDC 1, http://links.lww.com/JTO/A757) generated using Illumina Human Methylation 450K (HM450K) was obtained from TCGA.[31] First, DNA methylation level of 4415 CpG sites located in 182 previously reported TRGs (Supplementary

Table 3, SDC 1, http://links.lww.com/JTO/A757) was compared between ESCC and normal esophagus tissues. DNA methylation differences for each CpG site in regards to significance were visualized in a Volcano Plot. Terrain maps were utilized to visualize the distance generated by CpG methylation profiles for each sample. Finally, hierarchical clustering with bootstrapping analyses was employed to identify clusters of hypermethylated CpG sites on ESCCs specimens.

Detection of Gene-Specific Promoter Hypermethylation

Bisulfite-modified DNA was used as a template for separate PCRs with fluorescently labeled primer sets specific for methylated or unmethylated versions of six TRGs (Supplementary Table 1, SDC 1, http://links.lww.com/JTO/A757). PCRs were performed as previously described.[20] Amplifications were carried out as follows: 95°C for 3 min followed by 36–40 cycles of denaturation at 95°C (30 sec), annealing (30 sec), extension at 72°C (30 sec), and a final 72°C cycle (7 min extension).

PCR products were analyzed by capillary array electrophoresis (CEQ 8000XL; Beckman Coulter, Brea, CA) using Beckman Coulter WellRED dye-labeled phosphoramidites (Genset Oligos, La Jolla, CA) as previously described.[32] Leukocyte DNA from healthy donors were methylated in vitro with excess *Sss*I methyltransferase (New England BioLabs, Ipswich, MA) to generate methylated DNA as a universal methylated control.[33] Peripheral blood leukocytes DNA from healthy donors were amplified by *phi*-29 DNA polymerase and served as a universal unmethylated control. Each assay included ESCC specimens, two positive (DNA from a known methylated cell line and universal methylated control) and two negative (DNA from a known unmethylated cell line and universal unmethylated control) controls. Representative figures of TRG MSP are presented in Supplementary Figure 1 (SDC 2, http://links.lww.com/JTO/A758).

Biostatistical Analysis

The study was performed in concordance with the Reporting Recommendations for Tumor Marker Prognostic Studies of the National Cancer Institute.[34] To compare parametric variables, Student's t and ANOVA tests were used, while to compare nonparametric variables, Wilcoxon rank sum and Kruskal–Wallis tests were employed. Associations between categorical variables were assessed using the χ^2 or Fisher's exact tests. Trend analysis of LINE-1 unmethylated (or nonmethylated) status across AJCC stages was performed using the Cochran Armitage trend test. The Newman–Keuls multiple comparison test was used to determine whether each AJCC stage differed significantly in regards to methylation level. For the LINE-1 hypomethylation level-based discrimination between primary ESCC and adjacent normal mucosa, the receiver operating characteristics curve was generated and the area under the curve was calculated. Overall survival (OS) curves were generated using the Kaplan–Meier method and survival estimates were compared using the log-rank test for univariate survival analysis. In the univariate survival analysis, the LINE-1 U index, individual TRGs, and known prognostic factors such as depth of tumor invasion, pathological differentiation, AJCC stage, status of lymph node metastasis, or the number of metastatic nodes were examined. The variables that showed a trend of association to survival ($p < 0.1$) in the univariate analysis were entered into the multivariate Cox proportional hazard regression model. A stepwise selection method was used to obtain the final survival model. Proportional hazard assumption for the final model was tested using the Cox Snell residual plot. Biostatistical analyses were performed using SAS (version 9.2), and $p \leq 0.05$ was considered significant.

Genome-wide DNA methylation data was compared between groups using the Wilcoxon rank-sum test, and multiple comparisons were corrected using the Benjamini and Hochberg false discovery rate method. Unsupervised hierarchical cluster and terrain map analyses were performed using the Euclidean metric distance and the topology of the phenetic tree was evaluated by Bootstrap analysis with 100 iterations in MultiExperiment Viewer v4.9 (Dana-Farber Cancer Institute, Boston, MA).[35]

RESULTS

Patient Characteristics

The study included 114 ESCC eligible consecutive patients who underwent surgery with follow-up at Keio University Hospital from 1997 until 2002, and excluded ESCC patients with prior chemoradiotherapy. Table 1 summarizes

TABLE 1. Patients Demographics and Pathology Characteristics

Gender	
Male	106 (93%)
Female	8 (7%)
Age	
Mean ± SD	60.9 ± 7.46 (range, 39–81)
Primary tumor (T)	
T1	45 (39%)
T2	11 (10%)
T3	54 (47%)
T4	4 (4%)
Regional lymph nodes (N)	
N0	42 (37%)
N1	71 (62%)
Unknown	1 (1%)
AJCC stage	
Stage I	28 (25%)
Stage IIA	14 (12%)
Stage IIB	26 (23%)
Stage III	46 (40%)
Histological differentiation	
Well	27 (24%)
Moderate	72 (63%)
Poor	11 (10%)
Unknown	4 (3%)

AJCC, American Joint Committee on Cancer.

the clinicopathological variables of patients studied. The patient age at the time of diagnosis ranged from 39 to 81, with a median of 60.9 years. The ESCC patients were classified as follows: AJCC stage I (n = 28), stage IIA (n = 14), stage IIB (n = 26), and stage III (n = 46).

Global DNA Hypomethylation of ESCC is Higher than Normal Esophagus Epithelium

The LINE-1 U index level of ESCC specimens, including both primary tumors and metastatic lymph nodes (n = 140, mean = 0.52 ± 0.18), was significantly higher than adjacent normal esophageal epithelia (n = 28, mean = 0.11 ± 0.097; p < 0.0001). However, no difference was seen between primary tumors and metastatic lymph nodes (Fig. 1*A* and *B*). With respect to AJCC staging, LINE-1 U index level of greater than or equal to T2 tumors (n = 69, mean = 0.55 ± 0.18) was significantly higher than that of T1 tumors (n = 45, mean = 0.48 ± 0.15; p = 0.034; Fig. 1*C*). There was no significant association between LINE-1 U index and AJCC stages (data not shown). The receiver operating characteristics curve for primary ESCC compared with adjacent normal mucosa showed an area under the curve of 0.95 (Fig. 1*D*).

Genome-Wide DNA Methylation Analysis of ESCC and Normal Esophagus Identified Hypermethylated TRG

To detect aberrantly methylated TRGs in ESCC, genome-wide DNA methylation data was analyzed for 12 normal esophagus and 38 ESCC specimens (Supplementary Table 2, SDC 1, http://links.lww.com/JTO/A757). A total of 1631 CpG sites presented significantly (false discovery rate; p < 0.05) different methylation levels between normal and tumor tissues (Fig. 2*A*). Terrain maps generated with the methylation level of these 1631 CpG sites identified two independent clades containing ESCCs and normal tissues (Fig. 2*B*). Hierarchical cluster analysis using the 1631 CpG

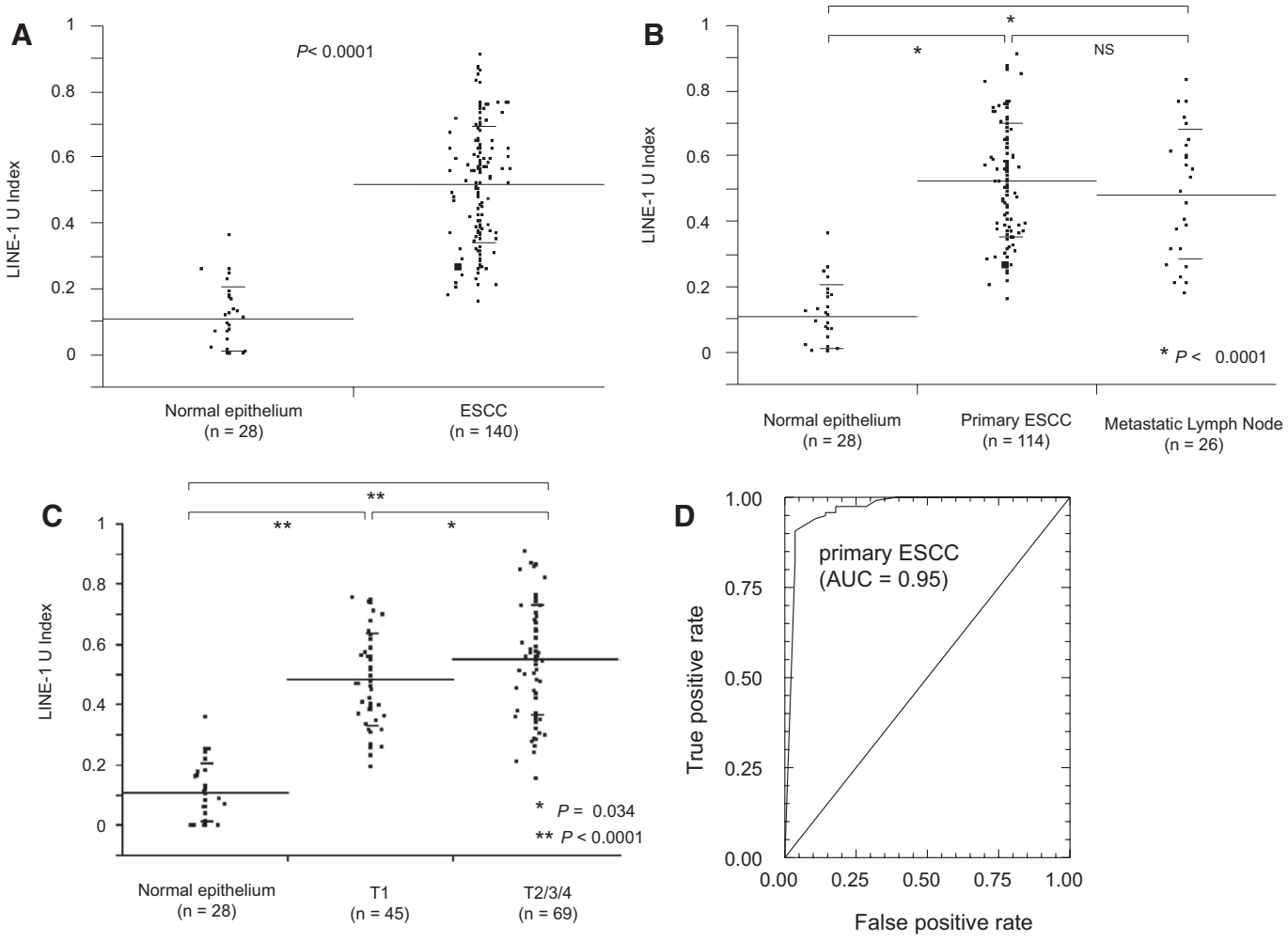


FIGURE 1. Comparison of LINE-1 U index between: *A*, Normal epithelium versus ESCC. *B*, Normal epithelium versus primary ESCC versus lymph node metastases. *C*, Normal epithelium versus T1 tumors versus T2/3/4 tumors. *p* values were obtained by Wilcoxon test. *D*, ROC curve for the discrimination between primary ESCC and adjacent noncancerous epithelium. AUC value reflecting group discrimination was indicated. ESCC, esophageal squamous cell carcinoma; LINE-I, long interspersed nucleotide element-1; ROC, receiver operating characteristics; AUC, area under the curve.

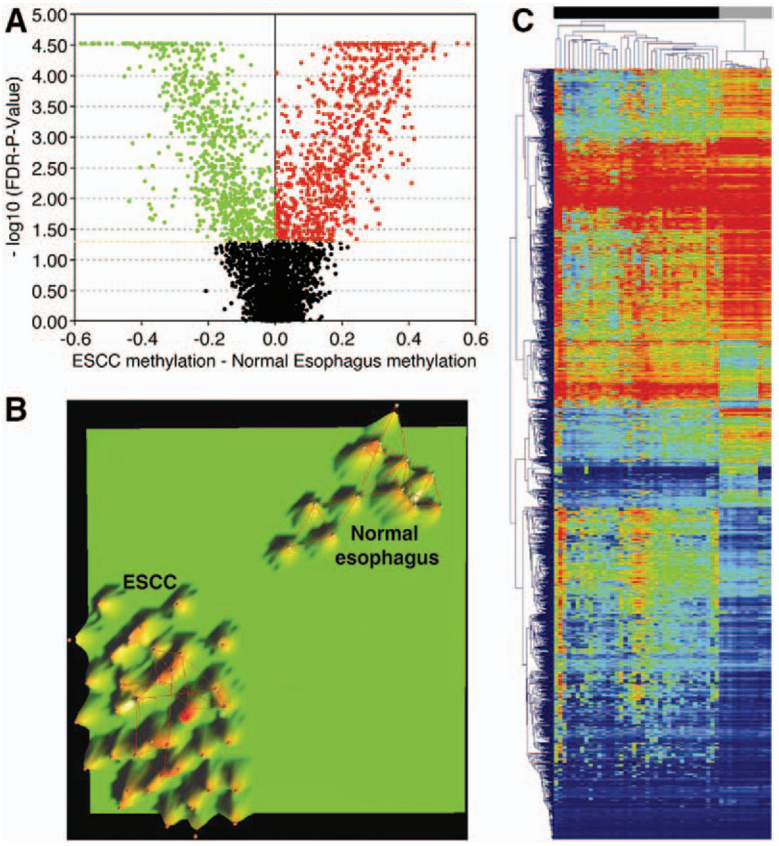


FIGURE 2. Genome-wide DNA methylation analysis of ESCC and normal esophagus tissues identifies differential methylation of 1631 CpG sites surrounding 160 TRGs. *A*, Volcano plot depicting CpG sites showing significant (*orange line*; FDR-p = 0.05) hypomethylation (*green dots*) and hypermethylation (*red dots*) in ESCC specimens. *B*, The terrain map analysis using Euclidean metric distance revealed a complete separation between normal tissues and ESCC. *C*, Unsupervised hierarchical cluster analysis. The methylation status in ESCC (*black bar*) and normal esophagus specimens (*grey bar*) were identified in two independent clusters (bootstrap = 100). ESCC, esophageal squamous cell carcinoma; TRG, tumor-related gene; FDR, false discovery rate.

sites identified two independent clusters (Fig. 2*C*). Among the most significantly hypermethylated CpG sites in ESCC, we identified six regions located in the promoter regions of *RARB*, *RASSF1*, *APC*, and *RUNX3* genes and in two different regions of the gene *CDKN2A*, one regulating the expression of the *p14ARF* and the other regulating *p16INK4a* isoforms.

Specific TRGs Hypermethylated in ESCC

To validate the hypermethylation of the identified regions, an independent large cohort of ESCC primary tumors (n = 114) was analyzed by targeted MSP. Promoter hypermethylation of *RARB*, *p16INK4a*, *RASSF1, APC*, *RUNX3*, and *p14ARF* were observed in 55%, 24%, 20%, 19%, 14%, and 8% of ESCC specimens, respectively (Table 2). The promoter CpG island regions of all six TRGs were unmethylated in adjacent normal esophageal epithelium. The frequency of promoter hypermethylation of *APC* was significantly higher in greater than or equal to T2 tumors (p = 0.024) and correlated with AJCC stage (p = 0.032), whereas the frequency of hypermethylation of all other TRGs was not significant.

We then stratified methylation status according to the distribution of hypermethylated regions of TRGs. Because of the low hypermethylation frequency, *p14ARF* gene was excluded from this analysis. The promoter CpG island methylation status was divided into two categories: high-frequency if greater than two TRGs were hypermethylated and low frequency if less than or equal to two TRGs were hypermethylated. Patients with missing methylation data for any of the TRGs were excluded from the analysis. Consequently, 14 patients were classified into high and 86 patients were classified into low-DNA methylation frequency (Table 3). There were no significant associations between the frequency of TRG hypermethylation and clinicopathological characteristics of ESCC.

Interestingly, an inverse correlation was detected between the global methylation level (assessed by LINE-1 U index) and the methylation of *RARB* gene. LINE-1 U index was significantly higher in the *RARB* hypermethylated group (n = 52, mean = 0.59 ± 0.17) than in the *RARB* unmethylated group (n = 42, mean = 0.48 ± 0.17; p = 0.0065; Fig. 3).

To assess possible variations on TRG DNA methylation statuses during ESCC progression to lymph node metastasis, a cohort of 20 available patients having lymph node metastases were analyzed. Overall, a high concordance on methylation status of all the TRGs was observed between primary tumors and paired lymph node metastases (greater than 70%). Yet, interestingly, de novo hypermethylation of *RUNX3* (15.8%), *RARB* (5.8%), and *p14ARF* (5%) were detected during the progression from primary to lymph node metastasis.

Survival Analyses of LINE-1 and TRGs Methylation Status in ESCC Patients

Log-rank test identified hypermethylation of *RASSF1* and *APC* genes to be significant predictors of OS. All patients with known methylation status for each marker were included in individual analyses. The unmethylated *RASSF1* group

TABLE 2. Correlation of CpG Island Hypermethylation with Tumor Progression

	RASSF1			*RARB*			*APC*			*p16INK4a*			*p14ARF*			*RUNX3*		
	Meth	Unmeth	*p* Value	Meth	Unmeth	*p* Value	Meth	Unmeth	*p* Value	Meth	Unmeth	*p* Value	Meth	Unmeth	*p* Value	Meth	Unmeth	*p* Value
T1 ($n = 45$)	8 (18%)	36		15 (48%)	16		4 (9%)	40		11 (23%)	34		4 (11%)	31		5 (12%)	38	
≥T2 ($n = 69$)	14 (21%)	52	NS	37 (59%)	26	NS	17 (26%)	49	0.024	16 (25%)	48	NS	4 (6%)	58	NS	10 (16%)	53	NS
Total ($n = 114$)	22 (20%)	88		52 (55%)	42		21 (19%)	89		27 (24%)	82		8 (8%)	89		15 (14%)	91	
Stage I ($n = 28$)	6 (22%)	21		8 (47%)	9		2 (8%)	24		5 (19%)	22		3 (15%)	17		3 (12%)	23	
Stage IIA ($n = 14$)	2 (15%)	11		7 (54%)	6		5 (36%)	9		4 (33%)	8		2 (15%)	11		1 (8%)	12	
Stage IIB ($n = 26$)	4 (16%)	21	NS	11 (55%)	9	NS	2 (8%)	24	0.032	7 (28%)	18	NS	2 (9%)	20	NS	2 (8%)	23	NS
Stage III ($n = 46$)	10 (22%)	35		26 (60%)	18		12 (27%)	32		11 (24%)	34		1 (2%)	41		9 (21%)	33	
Total ($n = 114$)	22 (20%)	88		52 (55%)	42		21 (19%)	89		27 (25%)	82		8 (8%)	89		15 (14%)	91	

($p = 0.0056$, median survival = 98 months versus 23.5 months, $n = 109$) and the unmethylated *APC* group ($p = 0.0069$, median survival = 41.5 months versus 15 months, $n = 109$) had significantly better OS than each respective methylated group (Fig. 4*A* and *B*). Patients were then divided into two groups based on the number of methylated genes (greater than two versus less than or equal to two, range: 0–4) among *RASSF1, RARB, p16INK4a, APC*, and *RUNX3* as outlined above, and survival association was examined. A higher number of methylated genes (>2 versus ≤2) was related to worse OS ($p = 0.0028$) by log-rank test (Fig. 4*C*), and showed 258% higher risk of death compared with those who had ≤ two methylated genes. A stepwise multivariate Cox regression analysis identified the number of metastatic lymph nodes, *RASSF1*, and *APC* to be significant predictors of OS ($p < 0.0001$). A proportionality test conducted on the data demonstrated that the proportional hazard assumption held true. As one metastatic node increased, the risk of death increased by 11% (HR = 1.108, 95% CI: 1.066–1.151; $p < 0.0001$), methylation of *RASSF1* increases risk of death by 78% (HR = 1.779, 95% CI: 1.002–3.159; $p = 0.049$), and methylation of *APC* increases risk of death by 108% (HR = 2.078, 95% CI: 1.143–3.775; $p = 0.016$) compared with the unmethylated group. LINE-1 hypomethylation level was not associated with survival outcome.

DISCUSSION

Recent studies have described epigenetic aberrations and their critical role in cancer progression and prognosis.[12,20] Most studies have focused on aberrant DNA methylation status of the promoter regions in TRGs and their silencing effects. Our study initially focused on significant differences in hypomethylation of LINE-1 between primary ESCC specimens and normal esophageal epithelia not correlating with AJCC stage and clinical outcome. Our results suggested that LINE-1 hypomethylation may be an early event during ESCC carcinogenesis and primary tumor development. This observation was supported by our finding that LINE-1 hypomethylation level was not significantly different between primary ESCC tumors and metastatic lymph nodes. A recent report also showed LINE-1 methylation levels of primary colorectal cancers are similar to those of matched liver and LN metastases, suggesting that LINE-1 hypomethylation may occur in early tumorigenesis and remains relatively stable during progression in colorectal cancers.[36] LINE-1 hypomethylation level of primary ESCC specimens was also significantly correlated with depth of tumor invasion. Although there was no significant correlation between LINE-1 hypomethylation and AJCC stage, this may be because in most ESCC cases lymph node metastases are frequently found, even in T1 tumors, when diagnosed and are classified into a worst prognosis group. In our series, 18 of 45 T1 tumors (40%) had lymph node metastases (classified into stage IIB), which is the most important prognostic factor in ESCC.[2] Prognostic significance was not observed regarding LINE-1 hypomethylation level in our study, which is inconsistent with a recent report demonstrating that LINE-1 hypomethylation of primary ESCC is significantly associated with disease-free survival and cancer-specific survival.[17] An explanation for this inconsistency is the

TABLE 3. Correlation of CpG Island methylation Status with Tumor Progression

	Total Numbers of Hypermethylated Genes				
	0	**1**	**2**	**3**	**4**
T stage analysis					
T1 (n = 39)	15 (38%)	17 (44%)	4 (10%)	3 (8%)	0 (0%)
≥T2 (n = 61)	14 (23%)	25 (41%)	11 (18%)	7 (11%)	4 (7%)
Total (n = 100)	29 (29%)	42 (42%)	15 (15%)	10 (10%)	4 (4%)
AJCC stage analysis					
Stage I (n = 23)	11 (48%)	8 (36%)	2 (8%)	2 (8%)	0 (0%)
Stage IIA (n = 13)	2 (15%)	6 (47%)	2 (15%)	3 (23%)	0 (0%)
Stage IIB (n = 23)	7 (30%)	11 (48%)	4 (17%)	1 (5%)	0 (0%)
Stage III (n = 41)	9 (22%)	17 (41%)	7 (17%)	4 (10%)	4 (10%)
Total (n = 100)	29 (29%)	42 (42%)	15 (15%)	10 (10%)	4 (4%)

AJCC, American Joint Committee on Cancer.

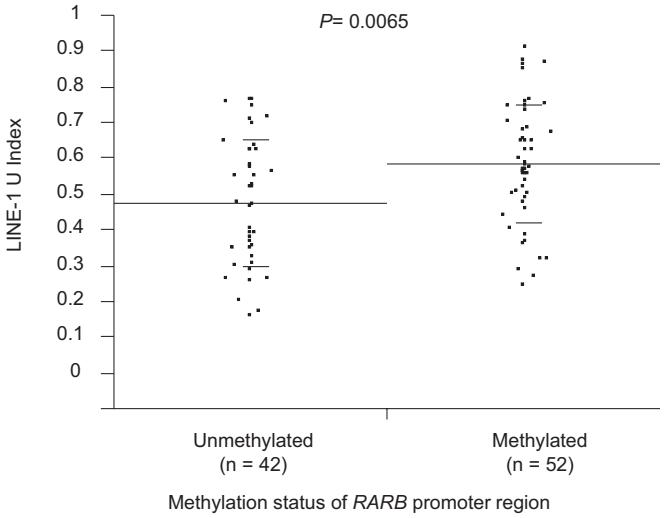


FIGURE 3. Comparison of LINE-1 U index between *RARB* hypermethylated group versus *RARB* unmethylated group. *p* values were obtained by Wilcoxon test. LINE-I, long interspersed nucleotide element-1.

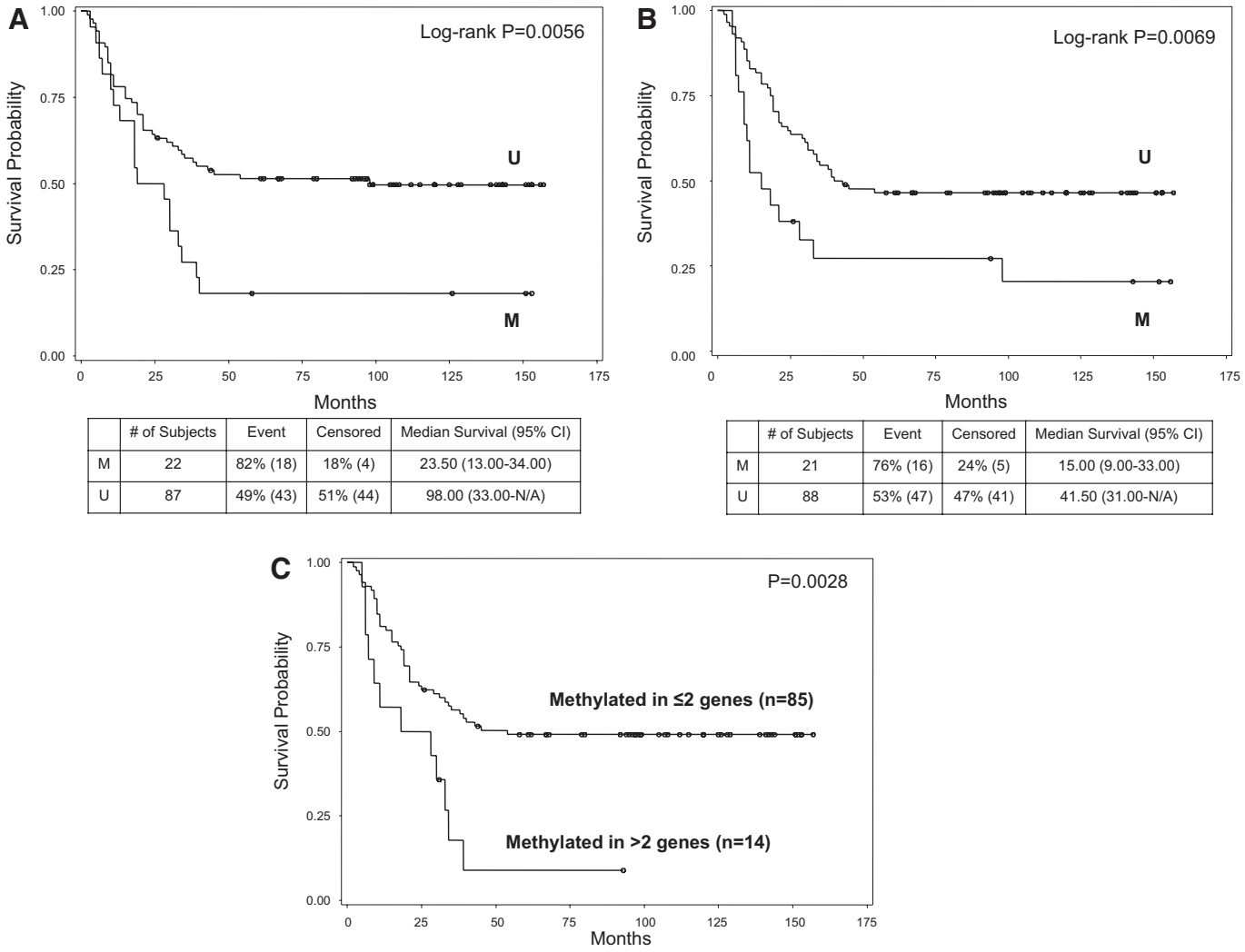
clear imbalance in the stage of patients studied. Iwagami et al. demonstrated that LINE-1 hypomethylation is associated with poor prognosis in ESCC; however, there were no significant differences when the analysis was restricted to advanced cases (stages II and III). Iwagami's study included only 54% of advanced cases, whereas our study was mostly composed (75%) of patients with advanced stages.

The number of epigenetic aberrations of multiple TRGs assessed by MSP has been correlated with increased cellular dysplasia in ESCC tumors.[37] In this regard, using TCGA data, we identified significant hypermethylation of six regions located in *RARB*, *RASSF1*, *APC*, *RUNX3*, *p16INK4a*, and *p14ARF* genes in ESCC. This observation was then validated in an independent large cohort of ESCCs using targeted MSP. We found that *APC* promoter hypermethylation correlates with depth of tumor invasion and AJCC stage. Previous studies investigating ESCC primaries demonstrated that *APC* promoter hypermethylation is a prognostic biomarker. However, the frequency of *APC* promoter hypermethylation was shown to be greater than 40%,[21,26,38] which is higher than the frequency detected in our study (19%). This discrepancy may be because of differences in the patient selection criteria, because in our cohort, all the advanced T4 cases treated with neoadjuvant chemotherapy were excluded. Our results indicated that patients with *APC* gene hypermethylation have a poorer survival rate. In addition, it has been reported that the down-regulation of both *RASSF1* transcripts and proteins expression in ESCC tissues is significantly associated with World Health Organization grade, tumor status, and lymph node metastasis.[39] In our study, *RASSF1* gene hypermethylation was also associated with poorer survival rates. Furthermore, our analyses also revealed that the number of hypermethylated genes was an independent predictor of shorter survival.

Our analysis was also extended to evaluate variations of DNA methylation during the progression to lymph node metastasis. The methylation of the TRG panel showed a high concordance between primary ESCC and lymph node metastases. However, two of these genes, *RARB* and *RUNX3*, presented hypermethylation status increase in lymph nodes in up to 29% and 26% of the cases, respectively. The observed enhancement on DNA methylation of this panel of TRGs may be a cause or a consequence of the lymph node metastasis. To elucidate this interesting question, a larger cohort of paired primary tumors and lymph node metastases will need to be assessed.

In addition to the clinical associations, we observed significant relation between epigenetic events. A significant inverse correlation between LINE-1 hypomethylation and *RARB* promoter hypermethylation was detected. This suggests that promoter hypermethylation of specific genes may be related to LINE-1 hypomethylation status. Hypomethylation status of LINE-1 in various cancers has been suggested to be related to genomic instability and aberrant hypermethylation of other TRGs.[20] However, this relation is not yet clearly understood.

Trimodality therapy or preoperative chemoradiotherapy followed by surgery is widely accepted as the standard of care



	# of Subjects	Event	Censored	Median Survival (95% CI)
M	22	82% (18)	18% (4)	23.50 (13.00-34.00)
U	87	49% (43)	51% (44)	98.00 (33.00-N/A)

	# of Subjects	Event	Censored	Median Survival (95% CI)
M	21	76% (16)	24% (5)	15.00 (9.00-33.00)
U	88	53% (47)	47% (41)	41.50 (31.00-N/A)

FIGURE 4. Kaplan–Meier curves of ESCC patients for OS. Patients are divided into subgroups according to: *A, RASSF1* promoter methylation status. *B, APC* promoter methylation status. *C,* The number of methylated TRGs (greater than two versus less than or equal to two). ESCC, esophageal squamous cell carcinoma; OS, overall survival; TRG, tumor-related gene.

for patients with locoregional esophageal squamous or adenocarcinomas.[40,41] Until recent years, surgical resection was the standard treatment for AJCC stage II/III ESCC; however, neoadjuvant chemotherapy with cisplatin plus 5-fluorouracil followed by surgery has currently emerged as a new standard treatment in Japan.[42,43] Importantly, the cohort of patients enrolled in our study did not receive any neoadjuvant therapy; therefore, the tumor tissues are considered to contain higher quality DNA because of avoidance of radiation/chemotherapy-induced DNA damage. Our findings of the significant association between LINE-1 and TRGs methylation status and ESCC may be useful for future postoperative treatment and management strategy decisions upon first diagnosis. For the future, biomarkers will be needed for prediction of preoperative chemoradiotherapy because this treatment approach is a currently accepted practice in most Asian countries.[42] Also, the identification of early risk factors of ESCC upon biopsy may be important as there is evidence of dietary prevention for reducing ESCC.[8,9] Epigenetic events are reversible; therefore, development of prevention protocols in patients who have high-risk precancerous or early stage ESCC may be important especially because ESCC becomes very difficult to treat once it progresses. Thus, early prevention strategies to prevent or reverse inactivation of TRGs may be beneficial.

Currently, there are no biomarkers to identify which patients are likely to be at high risk for aggressive ESCC. Discovery of such biomarkers is important for better management of patients and stratification for treatment purposes. Our results using a large number of samples implied that clinical tests of epigenetic aberrations of noncoding genomic regions LINE-1 and TRGs may serve as biomarkers for novel screening and clinical management of ESCC. Furthermore, there is potential to test these methylation biomarkers in endoscopic surveillance biopsies or serial serum collection after surgery to detect early ESCC or stratify patients who are at higher risk for recurrence. Future studies will involve validation of these epigenetic biomarkers in a multi-institutional treatment study.

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