Aberrant Methylation is Frequently Observed in Advanced Esophageal Squamous Cell Carcinoma

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Abstract. *The combined methylation status of the p16, helicase-like transcription factor (HLTF) and T-cadherin, H-cadherin (CDH13) genes in 35 resected primary esophageal squamous cell carcinoma (ESCC) was examined using methylation-specific PCR. To examine whether the methylation status could be a marker for the malignancy of ESCC, the methylation status was correlated with the clinicopathological features of the affected patients. Aberrant methylation of the p16, HLTF and CDH13 genes was detected in 28 (80%), one (3%) and five (14%) out of 35 ESCC specimens, respectively. ESCC with methylation-positive genes showed a trend towards larger tumor size and deeper invasion (p=0.139 and p=0.0664, respectively). Thus, the methylation of the p16, HLTF and CDH13 genes is a high malignancy factor in ESCC.*

Esophageal squamous cell carcinoma (ESCC) is a particularly aggressive cancer. Accumulating evidence has indicated that a series of genetic changes in dominant oncogenes, such as cyclin D1 and *hst1/int2,* and tumor suppressor genes, such as *p53* and *p16,* are involved in the pathogenesis of human ESCC (1-4). In addition, we found that several other genes are related to the pathogenesis of ESCC (5-7), indicating that investigation of genetic changes is important to clarify its tumorigenic pathway.

Several tumor suppressor genes contain CpG islands in their promoters, prompting many investigations into the role of methylation in silencing these genes. Numerous tumor suppressor genes show evidence of methylation silencing, providing a potential new pathway for their deactivation. A tumor suppressor gene, *p16*, was found to harbor promoter hypermethylation associated with the loss of protein expression in cancer cells (8). We previously examined the methylation status of the *p16* gene in 38 primary ESCC using methylation-specific PCR (MSP) (9). Aberrant promoter methylation of the *p16* gene was detected in 31 out of the 38 (82 %) ESCC studied.

The loss of expression of a helicase-like transcription factor (*HLTF*), a SWI/SNF family member gene, accompanied by *HLTF* promoter methylation was reported in colon cancer (10). Here, all colon cancer cell lines that lacked *HLTF* gene expression demonstrated methylation of CpG sites within the putative *HLTF* promoter, while methylation was not detected in the HLTF-expressing cell lines. In our previous study, *HLTF* methylation was detected in one out of 40 (3%) ESCC, suggesting that *HLTF* is not a common target for methylation and epigenetic gene silencing in ESCC (11).

Recently, it became clear that *CDH13* (H-cadherin, T-cadherin) expression is frequently silenced by aberrant methylation in colorectal cancer and adenoma (12). *CDH13* encodes a protein belonging to the cadherin family of cell surface glycoproteins responsible for selective cell recognition and adhesion (13). *CDH13* methylation was detected in five out of 37 (14%) ESCC, suggesting that *HLTF* is a common target for methylation and epigenetic gene silencing in ESCC and qualifies as a potential ESCC suppressor gene (14).

In this study, the combined methylation status of *p16*, *HLTF* and *CDH13*, in 35 resected primary ESCC was examined using MSP. To determine whether the methylation status could be a marker for the malignancy of ESCC, it was correlated with the clinicopathological features of the affected patients.

Materials and Methods

Sample collection and DNA preparation. Thirty-five primary tumors and corresponding normal tissues were collected consecutively

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Abbreviations: ESCC, Esophageal squamous cell carcinoma; MSP, methylation-specific PCR.

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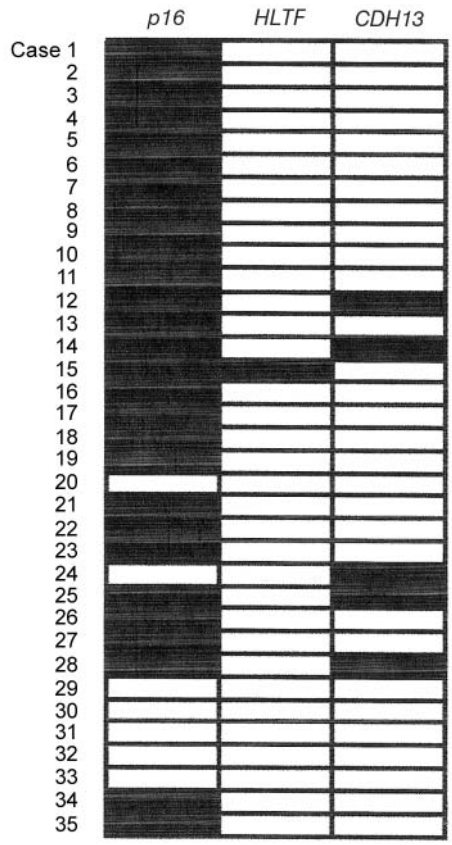

Figure 1. *Methylation status of the p16, HLTF and CDH13 genes in 35 ESCC. The methylated genes are shown as closed boxes.*

from colorectal cancer patients at Nagoya University Hospital, Japan. All the tissues were confirmed histologically. The collected samples were immediately stored at –80˚C until analysis. DNA was prepared as described previously (15).

Sodium bisulfite modification. One μg of genomic DNA extracted from tumor and corresponding normal colorectal tissue was subjected to bisulfite treatment, as described previously (9). Briefly, alkali-denatured DNA was modified by 2.1 M sodium bisulfite / 0.5 mM hydroquinone at pH5.0. The bisulfite-reacted DNA was then treated with NaOH, purified using the Wizard DNA Clean-Up System® (Promega, Madison, WI, USA), precipitated with ethanol and resuspended in distilled water.

MSP. The bisulfite-treated DNA was amplified using MSP. The primers and PCR conditions have been described previously (8, 10, 12). Ten μl of each PCR product was loaded directly onto non-denaturing 8% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis. The possible association between the number of methylated genes and clinicopathological features was examined using the Chi-squared test or Student's *t*-test. The computed *p*-values were two-tailed. Statistical significance was considered to be $p<0.05$.

Table I. *Clinicopathological features and the methylation status in ESCC patients.*

Clinicopathological features	Variables	No. of cases	Methylation status		*p*-value
			–	+	
Gender	male	30	5	25	0.855[a]
	female	5	1	4	
Age	17-90	35	66±9[c]	62±8	0.881[b]
Maximum tumor size	15-100 mm	35	41±9	50±20	0.139[b]
Invasion	≤pm[d]	12	4	8	0.0664[a]
	pm<	23	2	21	
Lymph node metastasis	–	15	3	12	0.698[a]
	+	20	3	17	
Deceased or alive	deceased	21	3	18	0.583[a]
	alive	14	3	11	
Total		35	6	29	

[a]Chi-squared test, [b]Student's *t*-test, [c]mean ± S.D., [d]proper muscle.

Results

The methylation status of the *p16*, *HLTF* and *CDH13* genes in ESCC and corresponding normal tissue specimens was examined using MSP, as described previously (9, 11, 14). The methylation status of the *p16*, *HLTF* and *CDH13* genes was initially examined in 38, 40 and 37 ESCC specimens, respectively. Thirty-five specimens were tested for the methylation status of all three genes. Aberrant methylation of the *p16*, *HLTF* and *CDH13* genes was detected in 28 (80%), one (3%) and five (14%) of the 35 ESCC specimens, respectively (Figure 1). All the tumors also exhibited lack of methylation of each gene, which might be the result of contamination by non-tumor cells in the tumor specimens. Otherwise, it is possible that these gene expressions had not been completely inhibited in the cancer specimens.

Then, the correlation between the methylation status and clinicopathological features was analyzed (Table I). There was no significant difference in the distribution of patients with methylated *p16*, *HLTF* and *CDH13* genes in terms of age, gender, lymph node metastasis, or prognosis. However, ESCC with methylation-positive genes showed a trend towards larger tumor size and deeper invasion ($p=0.139$ and $p=0.0664$, respectively).

Discussion

Our results indicated that large and invasive ESCC exhibited methylated genes including *p16*, *HLTF* and *CDH13*, making methylation of these genes a high malignancy factor. Shannon *et al.* also investigated the methylation status of *hMLH1*, *p16* and *MDR1* in 58 colorectal cancers (16). They

found methylation of these genes in 23%, 29% and 28% of colorectal cancer, respectively. As a result, these three genes were significantly methylated in microsatellite instability (MSI)+ colorectal cancer compared to MSI– cancer. Additionally, they were significantly associated with poor histological differentiation. Shannon *et al.*'s results agreed with ours in that aberrant methylation was closely related to the malignancy of the carcinoma.

In a prolonged search for novel markers to estimate the malignancy of ESCC, previously we have examined the *AIS* status in digestive tract cancer and found that all eight ESCC cell lines (100%) showed *AIS*/*TA-AIS* gene overexpression (7). Subsequently, *AIS* gene expression was tested in paired esophageal normal tissues and cancers. Twenty-five out of 39 primary ESCCs (64%) demonstrated an obviously higher expression of the *AIS* gene compared to paired normal tissues. Moreover, high *AIS* gene expression was significantly associated with lymph node metastases in ESCC ($p=0.0271$). These results suggested that *AIS* may be useful as a marker for advanced ESCC.

In an other study, we performed a quantitative RT-PCR for the *PAI-1* gene and evaluated the possible relationship between *PAI-1* gene expression levels and clinicopathological findings in ESCC (17). Significant increases in *PAI-1* scores were observed in metastasis-positive ESCCs (3.08 ± 0.80) compared to metastasis-negative ones (-0.31 ± 0.62) ($p=0.0042$). *PAI-1* expression scores significantly increased with the tumor stage ($p=0.05$; ANOVA). These results suggested that *PAI-1* might serve as a new parameter for predicting prognosis in ESCC.

ESCC, one of the most aggressive cancers, occurs at a high incidence rate in some countries including Japan. Treatment by surgery with subsequent chemotherapy and radiotherapy would benefit greatly from new genetic and epigenetic alteration parameters to estimate the malignancy.

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