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Aberrant methylation of Fragile Histidine Triad gene is associated with poor prognosis in early stage esophageal squamous cell carcinoma

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ABSTRACT

The aim of this study was to understand the clinicopathological and prognostic significance of promoter methylation of Fragile Histidine Triad (*FHIT*) gene in esophageal cancer. *FHIT* methylation in 257 primary esophageal squamous cell carcinomas was retrospectively analyzed by methylation-specific polymerase chain reaction. Aberrant methylation of *FHIT* was found in 85 (33%) of 257 esophageal cancer patients. The *FHIT* methylation was found to be significantly associated with exposure to tobacco smoke ($P = 0.007$) and with a poor prognosis in cases of stage 1–2 cancer irrespective of recurrence. The hazard of failure after esophagectomy for stage 1–2 cancers with *FHIT* methylation was about 5.81 (95% CI = 1.15–14.07; $P = 0.009$) times higher than in those without. Recurrence occurred in 116 (45%) of the 257 patients studied. The survival after recurrence in stage 1–2 cancers was also poorer for patients with *FHIT* methylation than in those without (HR = 2.31; 95% CI = 1.18–7.92; $P = 0.03$). In conclusion, aberrant methylation of the *FHIT* promoter was found to be significantly associated with exposure to tobacco smoke and with a poor prognosis for stage 1–2 cases, but not with recurrence rate. Our study suggests that *FHIT* promoter methylation may be an independent prognostic biomarker in early stage esophageal squamous cell carcinoma.

1. Introduction

Esophageal cancer is among the most fatal of human cancers, and worldwide 300 000 new cases are diagnosed annually; moreover its incidence is increasing in the Western world.[1–3] Although advances in perioperative management and standardization of surgical techniques have resulted in substantial reduction in the number of postoperative deaths after esophagectomy, prognosis after radical resection remains poor, regardless of disease stage. And, recurrence after curative resection is one of the major causes of this poor prognosis. Esophageal cancer recurs in approximately half of patients after a curative resection, and outcome for most of these patients is miserable.

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Fragile Histidine Triad (*FHIT*) is a tumour suppressor gene that spans the FRA3B common fragile site on 3p14.2, a site that frequently harbours chromosomal aberrations in many tumours, including esophageal cancer.[4–11] The *FHIT* gene is known to be involved in carcinogenesis of the esophagus. Aberrant transcripts of this gene were first described to be present in about 50% [5 of 10] of esophageal carcinomas.[7] Michael and colleagues[9] also reported homozygous deletions involving exon 5 of *FHIT* in 4 (20%) of 20 esophageal adenocarcinomas and hemizygous loss in 7 (35%) of 20 esophageal carcinomas. Loss of heterozygosity (LOH) in the *FHIT* region was also reported in 24 (55%) of 44 tumours.[12] In addition, Fhit protein was reported to be lost or reduced in 70–79% of esophageal squamous cell carcinomas,[13,14] and altered Fhit expression was detected in precarcinomatous lesions of the esophagus, which suggested that loss of Fhit expression may be an early event in the development of esophageal cancer.[9,13] Moreover, the inactivation of one *FHIT* allele in recombinant mice resulted in a much higher susceptibility to carcinogen-induced esophageal cancer.[15] Fhit protein expression has also been reported to be associated with esophageal squamous cell carcinoma progression.[14]

The aberrant methylation of CpG islands is an epigenetic change that induces the transcriptional silencing of tumour suppressor genes, and *FHIT* promoter is characterized by a CpG island in its 5′-untranslated region. Aberrant methylation of the 5′ CpG island of *FHIT* has been reported to be closely associated with transcriptional inactivation in esophageal squamous cell carcinoma,[16] and *FHIT* promoter methylation was found in approximately 15–45% of esophageal cancers and is now considered a major cause of Fhit expressional loss.[12,16,17] Thus, to further elucidate the role of Fhit in esophageal cancer, we studied the relationship between *FHIT* methylation and clinicopathological characteristics, especially recurrence and patient survival.

2. Materials and methods

2.1. Study population

A total of 257 patients who had been treated by curative surgical resection with histologically proven primary esophageal squamous cell carcinoma between May 1994 and February 2001 at the Department of Thoracic Surgery at the Samsung Medical Center (Seoul, Korea) participated in this study. Adenocarcinoma was not analyzed in the present study due to its low prevalence in Korea. The prevalence of squamous cell carcinoma and adenocarcinoma in Korea is about 98% and 1.5%, respectively. Patients who had a second primary esophageal cancer or those with R1, R2, or p-T4 suspicious of remnant malignancy were excluded from this study. Patients who had undergone preoperative adjuvant treatment or who died in the hospital after operation did not also participate in this study. Esophagectomy was performed with transhiatal esophagectomy for 12 patients (5%), 3-field lymphadenectomy (neck, mediastinal and abdominal) for 28 patients (11%) and 2-field lymphadenectomy (mediastinal and abdominal) for 217 patients (84%). Written informed consent for the use of paraffin-embedded tissues, as approved by the Institutional Board at the Samsung Medical Center, was obtained from all patients before operation.

Postoperative follow-up was scheduled at 1 month, and every 3 months for the first 2 years after esophagectomy, and then every 6 months for the next 2 years, and annually thereafter or more frequently if required. Chest X-ray, chest CT scan (lower neck to upper abdomen), carcinoembryonic antigen (CEA) and other serum chemistry were scheduled at every follow-up visit. If a patient was symptomatic, endoscopy, abdominal ultrasonography, brain MRI, and bone scan were performed. Whenever patients did not keep to the postoperative follow-up schedule, specialized nurses called on patients, and checked their health status. The median follow-up duration was 2.3 years.

Local (at the site of the primary tumour, the anastomotic site), regional (at lymph nodes or mediastinum, upper abdomen, and cervical area), and distant recurrences (in distant organs, pleura, or peritoneum) were evaluated by imaging or histologic/cytologic studies. A tumour was defined as "recurrent" if tumour occurred beyond one month after operation and was confirmed by histology/cytology or unequivocal radiologic proof. Lymph node metastasis was defined by following criteria: (1) greater more than 1 cm in short-axis diameter, or (2) marginal enhancement with central necrosis on CT, or (3) persistent enlargement during follow-up. With respect to tumour recurrence, among 116 patients with a recurrence, there were 66 (57%) with a locoregional recurrence and 50 (43%) with a distant recurrence. Recurrent tumours were treated by chemotherapy alone (75%), or radiotherapy alone (22%), or combined chemoradiotherapy (3%). The occurrence of death was evaluated as of May 31, 2004. Deaths due to recurrence were regarded as tumour-related deaths. The data on patients who died of causes other than esophageal cancer, did not die before the study end, or who were lost to follow-up during the study period were treated as censored data when calculating survival. TNM classification was determined by pathologic examination.

2.2. DNA extraction from paraffin blocks

Formalin-fixed, paraffin wax-embedded blocks containing at least 75% neoplastic tissues were cut into 10 μm-thick sections. Serial sections were placed on slides before DNA extraction and stained with hematoxylin–eosin to evaluate the admixture of tumourous/non-tumourous tissues. Areas corresponding to tumour were microdissected carefully, collected in 15 ml centrifuge tubes, and deparaffinized overnight at 63 °C in xylene. After centrifugation at 12000*g* for 5 min, supernatants were removed, and ethanol added to pellets (to remove residual xylene) and then removed by centrifugation. After ethanol evaporation, tissue pellets were resuspended in lysis buffer ATL (DNeasy Tissue kit, Qiagen) and the genomic DNA was isolated using a DNeasy Tissue Kit according to the manufacturer's instructions.

2.3. Methylation-specific polymerase chain reaction

The methylation status of the promoter region of *FHIT* was determined by Methylation-Specific PCR (MSP), as described by Herman and colleagues (Fig. 1A).[18] Two sets of primers

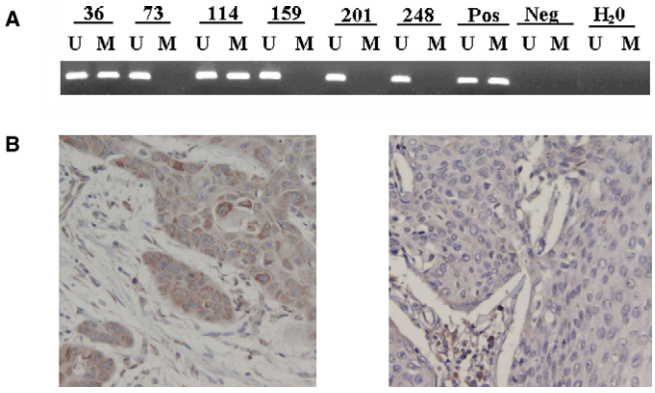

Fig. 1 – (A) Methylation analysis of the *FHIT* promoter in primary esophageal squamous cell carcinoma. Normal peripheral lymphocytes were used as negative controls for methylation, and in vitro methylated DNA was used as a positive control for methylation. The numbers shown are sample identification numbers. M = methylation-specific PCR; U = no methylation-specific DNA. (B) Examples of Fhit immunostaining in esophageal squamous cell carcinoma. A tumour was considered Fhit-positive if there was cytoplasmic reactivity in the neoplastic cells. Cancer cells show a diffuse Fhit-positive (left) and a Fhit-negative (right) staining. Magnification is 200×.

were designed, one specific for DNA methylated at the promoter region of *FHIT* gene and the other specific for unmethylated DNA. Primers for the methylated and unmethylated *HLTF* promoter sequences were previously published by Kuroki and colleagues.[12] The primer sequences used to amplify the methylated *FHIT* gene were 5′-TTGGGG-CGCGGGTTTGGGTTTTTACGC-3′ (sense) and 5′-CGTAAACGA-CGCCGACCCCACTA-3′ (antisense), and the primer sequences used for unmethylated *FHIT* were 5′-TTGGGGTGTGGGTTT-GGGTTTTTATG-3′ (sense) and 5′-CATAAACAACACCAACCCC-ACTA-3′ (antisense). DNA from peripheral blood lymphocytes of a healthy individual was treated with SssI methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and used as a positive-control for methylated alleles. Bisulfite-modified DNA from normal lymphocytes served as a positive control for unmethylated alleles, and unconverted DNA from normal lymphocytes was used as a negative control for methylated alleles. Negative control samples without DNA were included in each PCR set.

2.4. *Immunohistochemical analyses of fhit*

Formalin-fixed and paraffin-embedded tissues in 5 μm-thick sections were deparaffinized in xylene and rehydrated through a series of alcohols. For the purpose of antigen-retrieval, the sections were treated in 10 mmol/l citrate buffer (pH 6.0) for 10 min at 100 °C in a pressure-cooker. The sections were incubated with rabbit polyclonal antibody to human Fhit (Zymed Laboratories Inc., South San Francisco, CA) at 1:100 dilution in blocking solution overnight at 4 °C, and then treated with biotinylated secondary antibody (Histostatin-SP, Zymed) and incubated for 30 min in streptavidin-HRP (Histostatin-SP, Zymed), and 3.3′-diaminobenzidine tetrahydrochloride was used as the chromogen. All sections were counterstained with hematoxylin. A tumour was considered Fhit-positive if there was cytoplasmic reactivity in neoplastic cells (Fig. 1B).

2.5. *Statistical analysis*

The Wilcoxon rank sum test and Fisher's exact test (or the χ^2 test) were used to analyze continuous and categorical variables by univariate analysis, respectively. Survival duration was calculated from the date of surgery or recurrence until death. The effect of *FHIT* methylation on time to death was estimated using the Kaplan–Meier method, and the significance of differences in survival between two groups was evaluated using the log-rank test. Cox proportional hazards regression analysis was used to estimate the hazard ratios of independent factors for survival, after controlling for potential confounding factors such as age, sex, number of positive lymph nodes, and smoking status. All statistical analyses were two-sided, with a 5% type I error rate.

3. Results

3.1. *Clinicopathologic characteristics*

The associations between *FHIT* methylation and clinicopathologic features are listed in Table 1. Aberrant methylation of *FHIT* promoter was detected in 85 (33%) of 257 cases with esophageal squamous cell carcinoma. Mean overall patient age was 61 years and those without *FHIT* methylation were a little younger than those with *FHIT* methylation, but this difference was not statistically significant ($P = 0.41$). *FHIT* methylation was found at similar frequencies in men and women ($P = 0.62$). *FHIT* methylation was found to be significantly associated with pack-years smoked ($P = 0.007$), and those with *FHIT* methylation were more likely to be current smokers than those without ($P = 0.01$). No significant relationship was found between *FHIT* methylation and pathologic stage ($P = 0.37$), or with T, N, and M stages (data not shown). Esophageal cancer occurred more frequently in the mid-esophagus (45%) than in the upper-esophagus (14%) and lower-esophagus (39%), but no relationship was found between *FHIT* methylation and esophageal cancer location ($P = 0.47$), or be-

Table 1 – Clinicopathologic characteristics of the 257 patients with esophageal squamous cell carcinoma

Variables	FHIT methylation		P-value
	Absent (*n* = 172)	Present (*n* = 85)	
Age[a]	61 ± 8	62 ± 9	0.41
Sex			
Male	159	80	
Female	13	5	0.62
Packyears[a]	53 ± 41	67 ± 39	0.007
Smoking status			
Never	22	3	
Former	91	38	
Current	59	44	0.01
Pathologic stage			
1	28	13	
2	60	37	
3	55	19	
4	29	16	0.37
Location of tumour[b]			
Cervical	5	0	
Upper	23	10	
Middle	78	39	
Lower	64	36	0.47
Lymphatic invasion[b]			
Absent	154	73	
Present	16	11	0.37
Vascular invasion[b]			
Absent	166	79	
Present	5	5	0.13
Perineural invasion[b]			
Absent	166	83	
Present	5	1	0.27

a Mean ± standard error.
b Tumour location, lymphatic, vascular, and perineural invasion data are missing for 2, 3, 2, and 2 patients, respectively.

tween *FHIT* methylation and vascular/lymphatic/perineural invasions. Moreover, number of positive lymph nodes was not associated with *FHIT* methylation ($P = 0.65$).

3.2. *FHIT methylation and the recurrence of esophageal cancer*

Esophageal cancer recurred in 116 (45%) of the 257 patients studied, and we investigated the relationship between prevalence of recurrence and the *FHIT* methylation (Fig. 2). The disease was found to recur more frequently in patients with advanced stage at the time of esophagectomy ($P = 0.004$; Fig. 2A). Recurrence occurred in 30% of those with stage 1 and in 38% of those with stage 2, and in those with stages 3 and 4, the prevalences of recurrence were 52% and 62%, respectively. The incidence of tumour recurrence was not found to be associated with *FHIT* methylation by crude data analysis ($P = 0.37$). Because tumour recurrence was found to be significantly associated with pathologic stage, we stratified the data according to pathologic stage and reanalyzed the relationship between *FHIT* methylation and the development

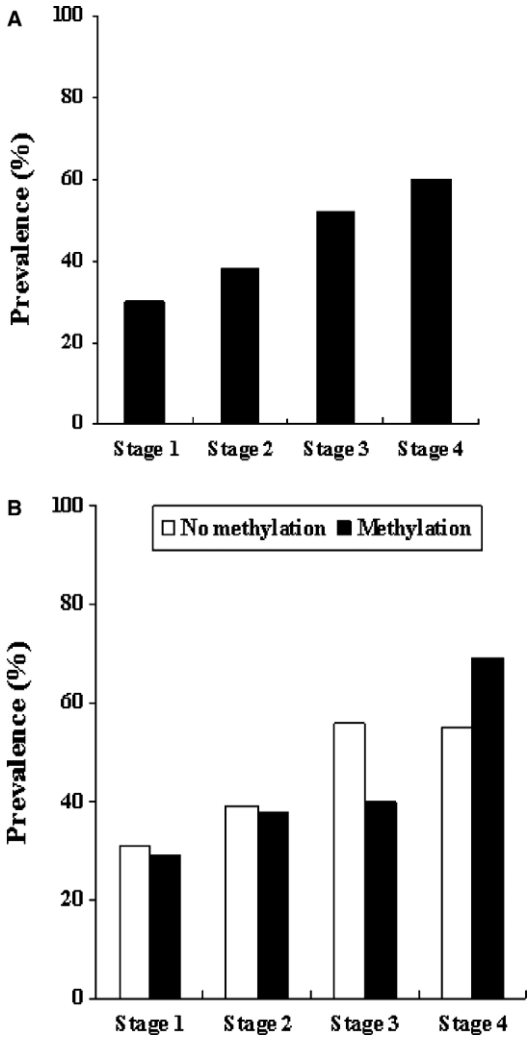

Fig. 2 – Prevalence of recurrence in the 257 esophageal squamous cell carcinomas. (A) The prevalence of recurrence was found to increase with pathologic stage, which suggests the strong association between recurrence and pathologic stage ($P = 0.004$). (B) Because of strong relationship between tumour recurrence and pathologic stage, the data was stratified according to pathologic stage for the analysis of relationship between *FHIT* methylation and the development of recurrence. The prevalence of recurrence between patients with and without *FHIT* methylation was not significantly different in each stage, suggesting no relationship between recurrence rate and *FHIT* methylation.

of recurrence. However, no relationship was found between *FHIT* methylation and recurrence, irrespective of pathologic stage (Fig. 2B), which suggest that *FHIT* methylation was not associated with recurrence rate.

3.3. *FHIT methylation and long-term overall survival after esophagectomy*

Before analyzing the effect of *FHIT* methylation on long-term overall survival, we initially measured overall survival in the 257 patients. The surgery-related in-hospital death rate of

all 257 patients was 3.9%. The 1-, 2-, 3-, and 5-year survival rates after esophagectomy for all patients were 74%, 62%, 47%, and 41%, respectively. The 5-year survival rate was 40% in men and 43% in women ($P = 0.37$). Next, we investigated the effect of *FHIT* methylation on overall survival in the 257 patients (Fig. 3). Data was stratified by disease stage, a recognized independent risk factor in esophageal cancer. *FHIT* methylation was found to be significantly associated with overall survival in the early stage of esophageal squamous cell carcinoma. For the 138 stage 1–2 patients, overall 3-year survivals after esophagectomy were 52% and 66% for those with and without *FHIT* methylation, respectively ($P = 0.03$; Fig. 3A). For the 119 stage 3–4 patients, overall 3-year survivals after esophagectomy were similar in those with and without *FHIT* methylation (37% and 32%, respectively; $P = 0.51$; Fig. 3B). Recurrence-free survival was also found to be significantly poorer in stages 1–2 cases with *FHIT* methylation than those without (data not shown).

3.4. *FHIT methylation and overall survival of patients without recurrence*

FHIT methylation was found to be associated with the overall survival in the early stage of the disease (Fig. 3), but not with recurrence rate (Fig. 2). Finally, we investigated the effect of *FHIT* methylation on patient survival according to recurrence, since recurrence is a known independent prognostic factor and the prognosis of patients with recurrent esophageal cancer is known to be extremely poor. Therefore, the data was further stratified according to the presence or absence of recurrence, and the effect of *FHIT* methylation on survival was reevaluated in patients with recurrence and those without, separately.

Of the 141 patients that did not experience recurrence, survival showed no relation with *FHIT* methylation in pathologic stages 3–4 ($N = 54$), whereas *FHIT* methylation was found to be significantly associated with a poor prognosis in the stage 1–2 patients ($N = 87$); 3-year survivals of those with and without *FHIT* methylation among these 87 patients were 67% and 81%, respectively ($P = 0.05$). However, the 3-year survival for 54 stage 3–4 patients with and without *FHIT* methylation were 47% and 53%, respectively, and this difference was not statistically significant ($P = 0.29$). These data indicate that *FHIT* methylation was significantly associated with overall survival in the early stage cases without recurrence.

3.5. *Survival and FHIT methylation in recurrent cases*

Patient survival in recurrent cases was analyzed in three ways: (1) long-term overall survival after esophagectomy (Fig. 4A and B), (2) survival after recurrence (Fig. 4C and D), and (3) time-to-recurrence (Table 2). Survival after esophagectomy or recurrence in recurrent stage 1–2 cases were first examined with respect to *FHIT* methylation status. The median survival after esophagectomy in recurrent stage 1–2 cases with and without *FHIT* methylation were 23 and 35

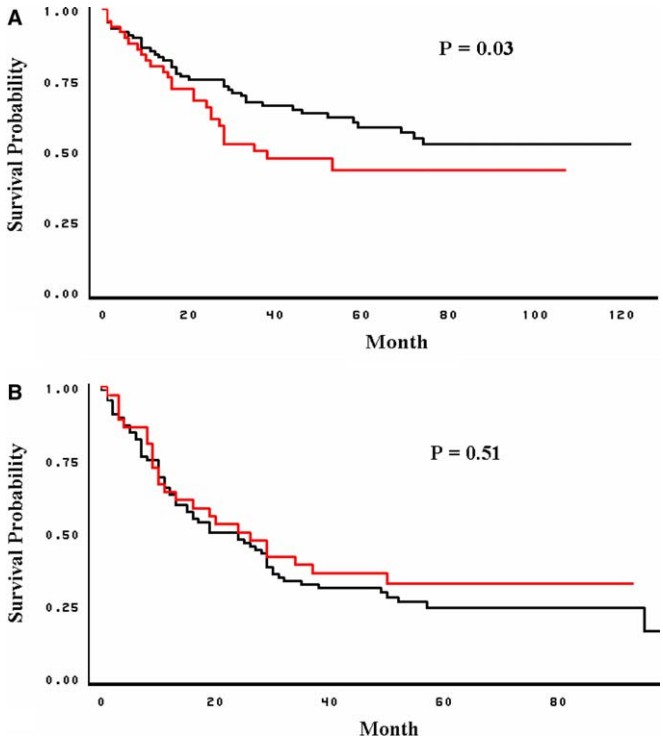

Fig. 3 – Kaplan–Meier plots of overall survival after esophagectomy in the 257 patients. (A) Patients with hypermethylation of the *FHIT* promoter had poorer survival compared with those without hypermethylation of *FHIT* promoter in stages 1–2 ($n = 138$). This difference was statistically significant based on Log-rank test ($P = 0.03$). (B) However, the difference was not significant in stages 3–4 ($n = 119$) ($P = 0.51$). The red and black lines denote those with and without *FHIT* methylation, respectively.

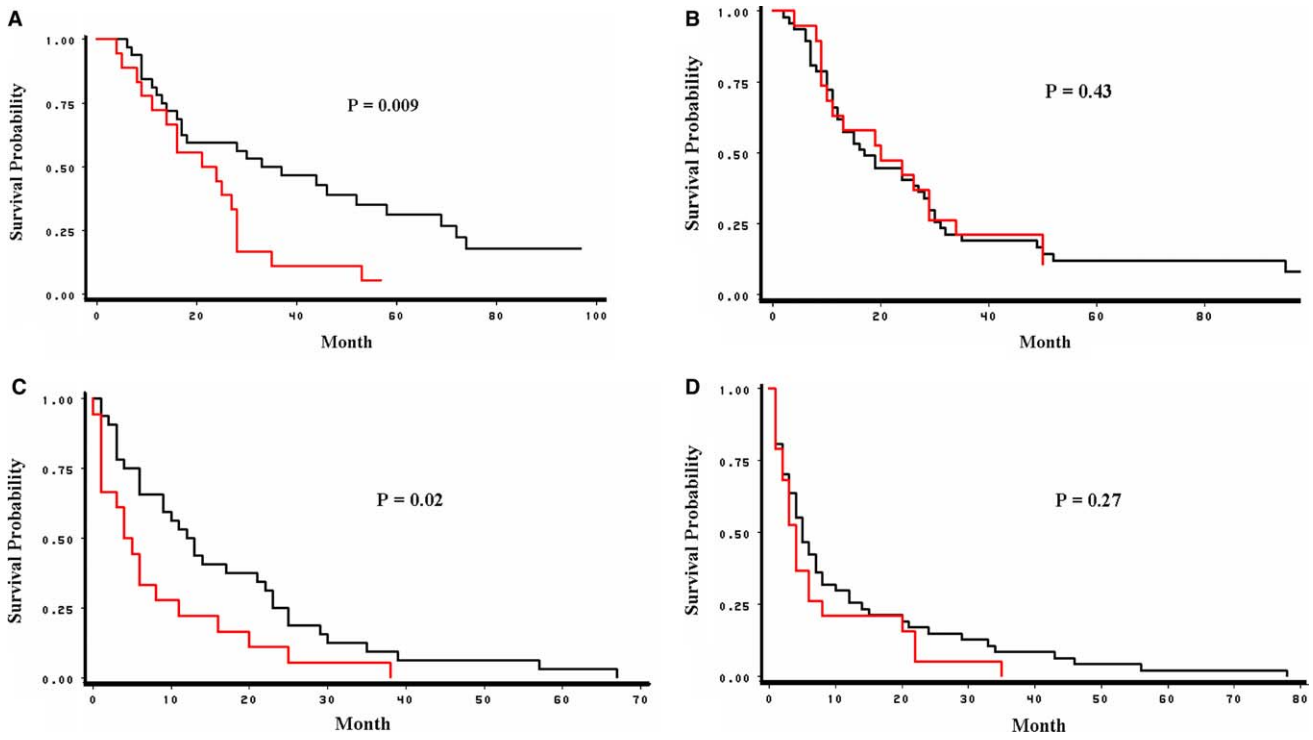

Fig. 4 – Kaplan–Meier plots of survival after esophagectomy and recurrence in the 116 patients with recurrent esophageal squamous cell carcinoma. Overall survival after (A) esophagectomy and (C) recurrence in the 50 recurrent stages 1–2 cases was poorer in patients with *FHIT* methylation compared with those without *FHIT* methylation. However, the survival after (B) esophagectomy and (D) recurrence in the 66 recurrent stages 3–4 cases was not significantly different. The red and black lines denote those with and without *FHIT* methylation, respectively.

Table 2 – Relationship between time-to-recurrence and FHIT methylation in the 116 patients with recurrent esophageal cancer

	Time-to-recurrence[a]			P-value
	1 year <	1–2 years	>2 years	
Stage 1–2				
FHIT methylation				
No	15	7	10	
Yes	11	7	0	0.02
Stage 3–4				
FHIT methylation				
No	28	13	6	
Yes	11	3	5	0.38

a Interval between esophagectomy and recurrence.

months, respectively (Fig. 4A). The median survival time after recurrence for stage 1–2 patients with *FHIT* methylation was 5 months and for those without *FHIT* methylation 13 months ($P = 0.02$; Fig. 4C). For 66 recurred stage 3–4 patients with and without *FHIT* methylation the median survival after esophagectomy and recurrence were similar (Fig. 4B and D). Moreover, time-to-recurrence in stage 1–2 patients was found to be correlated with survival duration after recurrence ($r = 0.34$, $P = 0.03$) and after esophagectomy ($r = 0.82$, $P = 0.001$).

The relationship between *FHIT* methylation and time-to-recurrence (the interval between esophagectomy and recurrence) after esophagectomy was summarized according to pathologic stage in the 116 patients that experienced recurrence (Table 3); 65 (57%) of these 116 recurred within 1 year of surgery, and 95 (83%) within 2 years. Of the 50 stage 1–2 cancers that recurred, 26 (52%) recurred within 1 year, and 14 (28%) between 1 and 2 years, and 10 (20%) after 2 years postoperatively. Time-to-recurrence for stage 1–2 cancers was found to be significantly associated with *FHIT* methylation ($P = 0.02$): 15 (47%) of the 32 stage 1–2 cases without *FHIT* methylation recurred within 1 year and 7 (22%) between 1 and 2 years, whereas of 18 cases with *FHIT* methylation 11 (61%) recurred within 1 year and 7 (39%) between 1 and 2 years postoperatively. In addition, recurrence occurred after 2 years in 10 (31%) of 32 patients without *FHIT* methylation, whereas no recurrence occurred after 2 years postoperatively in patients with *FHIT* methylation. Of the 66 patients in pathologic stage 3–4 that experienced recurrence no relationship was found between time-to-recurrence and *FHIT* methylation ($P = 0.38$).

3.6. *Cox proportional hazard analysis*

Stratified Cox proportional hazards regression analysis was performed according to pathologic stage to determine whether *FHIT* methylation was an independent risk factor of overall survival, after controlling for age, sex, recurrence

Table 3 – Stratified Cox proportional hazards analysis in esophageal squamous cell carcinomas (*n* = 257)

	HR[a]	95% CI[a]	P-value
Stage 1–2 (*n* = 138)			
FHIT methylation			
No	1.00		
Yes	5.81	1.15–14.07	0.009
Stage 3–4 (*n* = 119)			
FHIT methylation			
No	1.00		
Yes	1.28	0.89-3.51	0.46

*Adjusted for age, sex, recurrence treatment (yes/no), location of tumours, the site of recurrence (locoregional and distant), exposure to tobacco smoke, number of involved lymph nodes, operative method (transhiatal, 2-field, and 3-field), lymphatic/vascular invasion, and the presence of recurrence.
a HR; hazard ratio, CI; confidence interval.

treatment (yes/no), location of the tumours, the site of recurrence (locoregional and distant), exposure to tobacco smoke, number of involved lymph nodes, operative method (transhiatal, 2-field, and 3-field), lymphatic/vascular invasion, and the presence of recurrence (Table 3). The hazard of failure after esophagectomy for stage 1–2 cases with *FHIT* methylation was about 5.81 (95% CI = 1.54–14.07; $P = 0.009$) times higher than those without *FHIT* methylation. However, survival after esophagectomy in stage 3–4 patients was not associated with *FHIT* methylation (HR = 1.28; 95% CI = 0.39–4.81; $P = 0.54$).

Stratified Cox proportional hazard regression analysis was also performed in recurred stage 1–2 cases to determine whether *FHIT* methylation was an independent risk factor of survival in recurred cases, after controlling for age, sex, adjuvant therapy, location of the tumours, the site of recurrence, exposure to tobacco smoke, number of involved lymph nodes, operative method, lymphatic/vascular invasion (data not shown). The hazard of failure after esophagectomy for recurred cases with *FHIT* methylation was 3.02 (95% CI = 1.54–10.32; $P = 0.01$) times higher than those without *FHIT* methylation. Survival after recurrence was also poorer in patients with *FHIT* methylation (HR = 2.31; 95% CI = 1.18–7.92; $P = 0.03$). These data suggest that *FHIT* methylation may be an independent prognostic factor in the early stage of squamous esophageal carcinoma, irrespective of recurrence.

4. Discussion

The long-term survival of patients with esophageal cancer remains poor because of the high incidence of lymph node metastasis and early recurrence after curative operation. In the present study, 82% of tumours recurred within 2 years of curative resection, and 62% of these patients died from the disease within 2 years of recurrence, indicating that recurrence is a major prognostic factor. Thus, recurrence prevention is potentially important for improving patient survival.

The prevalence of *FHIT* methylation in the present study is consistent with the findings of other groups.[12,16,17] Smoking is known to contribute to esophageal carcinogenesis.[3,19] In the present study, aberrant methylation of the *FHIT* gene was significantly associated with exposure to tobacco smoke, which supports previous findings that the *FHIT* gene is targeted by carcinogens in cigarette smoke, and that the frequency of *FHIT* abnormalities at the DNA or protein level increases in smokers.[20–29] Moreover, the loss of *FHIT* allele is known to increase susceptibility to carcinogen-induced tumour development, including that of esophageal cancer.[15,30] Based on these observations, it is apparent that *FHIT* methylation, due perhaps in the large part to tobacco smoke exposure, contributes to the development of esophageal squamous cell carcinoma. Recently Shimada and colleagues[14] reported that the loss of Fhit protein expression in esophageal squamous cell carcinoma is not associated with a smoking history. Such variations between observations may be due to Fhit protein reduction by other causes rather than *FHIT* methylation or a small sample size. Accordingly, further work is required to evaluate the relationship between the *FHIT* methylation and exposure to tobacco in a large cohort.

The role of the *FHIT* gene in esophageal cancer has been investigated by several groups. The absence or marked down-regulation of Fhit protein expression has been reported in preneoplastic lesions of esophagus. Fhit expression was lost in 8 of 12 carcinomas in situ, in 2 of 4 severe dysplasias, in 4 of 8 moderate dysplasias, and in 3 of 9 mild dysplastic lesions.[13] Kitamura and colleagues[31] also found significant loss or reduction of Fhit expression in 13 of 19 (68.4%) CIS lesions, and in 10 of 23 (43.5%) dysplastic lesions. Moreover, alteration of *FHIT* at the level of DNA or RNA was reported in premalignant and normal tissues. Alterations of Fhit transcripts were observed in 12 (86%) of 14 Barrett metaplasia,[9] and *FHIT* methylation was found in 30% of corresponding noncancerous tissues of esophageal squamous cell carcinomas.[12] In the present study *FHIT* methylation was not found to be associated with pathologic stage and was detected in 33% and 39% of stages 1 and 2 cancers, respectively. These observations suggest that Fhit may play an important role during the early stage of esophageal carcinogenesis, although it is not currently clear how alterations in *FHIT* contribute to the tumour phenotype.

Aberrant methylation of the *FHIT* gene has been analyzed in several epithelial cancers including lung, renal, head and neck, esophageal and breast carcinomas. However, the prognostic value of *FHIT* methylation in these tumours remains unclear. Moreover, few data are available on the effects of *FHIT* methylation on the prognosis of esophageal cancer.[14] The present data demonstrates that *FHIT* methylation has an adverse effect on the prognosis of early stage esophageal squamous cell carcinomas. However, it is unclear how the aberrant methylation of the *FHIT* gene influences the survival of patients with early stage esophageal squamous cell carcinoma. Since *FHIT* was first cloned by positional cloning in 1996,[32] many have suggested its role as a tumour suppressor;[30,33–42] however, not all studies reported tumour suppression by Fhit in transfected cancer cells.[43–45] In addition, Fhit re-expression in Fhit-negative cells lacking endogenous Fhit protein expression has been shown to suppress tumour formation by at least in part cell cycle control and apoptosis induction in a variety of human cancer cells, but the results obtained have been somewhat conflicting.[28,34–46] The induction of apoptosis and the abolition of tumourigenicity by *FHIT*

gene transfer in esophageal cancer was reported to be in part caspase-dependent.[37] And, the transfection of *FHIT* into a H460 lung cancer cell line lacking Fhit protein expression induced cell cycle arrest at G_0–G_1, possibly via the up-regulation of the cell cycle inhibitor $p21^{waf1}$ protein.[35] In addition, the adenoviral transduction of *FHIT* gene into Fhit-negative esophageal cancer cell lines resulted in the accumulation of cells at S to G_2-M, with the suppression of cell growth in vitro with some apoptosis.[38] On the other hand, overexpression of Fhit in dividing HeLa cells did not alter cell cycle kinetics or inhibit cell proliferation.[43] Recently, Cavazzoni and colleagues[42] reported a dose-dependent effect of Fhit-inducible expression on cellular proliferation in a Calu-1 lung cancer cell line. They found an accumulation of cells in the G_0/G_1 phase and an up-regulation of $p21^{waf1}$, but no apoptotic sub-G_1 peak. These conflicting results may indicate that: (1) Fhit functions at a particular point during the multistage carcinogenic process; (2) susceptibility to cell growth inhibition by Fhit is dependent on cell type; and (3) the transfected gene is expressed at different levels, or that the endogenous levels of Fhit protein are a cause of observed differences.

To demonstrate that *FHIT* promoter methylation leads to loss/reduction of Fhit protein expression, we analyzed the pattern of Fhit protein using immunohistochemistry. Only 98 paraffin blocks were available for immunohistochemistry. We observed that 66% (65 of 98) was Fhit-negative in immunohistochemistry and that all samples with hypermethylation of *FHIT* promoter were Fhit-negative in immunohistochemistry. So we believe that *FHIT* promoter methylation is functional in this study. Considering all of the evidence above, and the short interval between esophagectomy and recurrence in early stage cancers showing *FHIT* methylation, and the absence of a relationship between *FHIT* methylation and the prevalence of recurrence after esophagectomy in the present study, it is reasonable to speculate that Fhit affects the prognosis by controlling cellular growth rates of cells responsible for survival after esophagectomy. Unfortunately, our study is limited by the lack of adenocarcinoma, and additional studies in a larger patient population including adenocarcinoma that are required to further understand the biological significance of Fhit loss in esophageal cancer. In the present study, prognosis after esophagectomy was poorer in the patients with recurrence than those without recurrence. In addition, time-to-recurrence was shorter in recurrent stage 1–2 cases with *FHIT* methylation than in those without, and was found to be strongly correlated with overall survival after esophagectomy in patients that experienced recurrence, which suggests that treatment with a suitable demethylating agent may improve the survival of esophageal squamous cell carcinoma patients with a high risk of recurrence. More studies targeted at identifying reliable predictors of recurrence is needed prior to a clinical trial of demethylating agent in esophageal squamous cell carcinoma. In summary, our study suggests that aberrant methylation of the *FHIT* gene is significantly associated with poor survival in those with early stage esophageal squamous cell carcinomas.

Conflict of interest statement

None declared.

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