

Altered Fhit Expression and Its Relationship with p53 Overexpression in Non-small Cell Lung Cancers

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Background : *FHIT* (Fragile histidine triad), the tumor suppressor gene at 3p14.2, encompasses the FRA3B fragile site and is a common target of deletions in primary human epithelial cancers, including those of the lung, head and neck, stomach, cervix, breast, and kidney. We investigated the association of Fhit expression with clinicopathologic features, including smoking history, and tried to correlate its altered expression with p53 overexpression in 45 non-small cell lung cancers. **Methods :** Immunohistochemical staining was performed on the paraffin sections, using primary anti-GST-Fhit and anti-human p53 antibodies. A four-tiered scoring system, incorporating both intensity of staining and the percentage of cells stained was used. Composite scores ≤ 3 were defined as a marked reduction or loss of Fhit or p53 protein expression. **Results :** Among the 45 tumors analyzed, 35 (77.8%) were markedly reduced or negative for Fhit protein expression. The reduced expression of Fhit protein was found to be significantly higher in smokers than in nonsmokers and also higher in squamous carcinoma compared with adenocarcinoma. Fhit and p53 alterations were found to be independent events, because there was no significant difference of Fhit-negativity between p53-positive and -negative groups. **Conclusion :** These results indicate that the Fhit alteration preferentially occurs in smokers and in the squamous type of non-small cell lung carcinomas. In addition, the results support the notion that Fhit alterations play an important role in the pulmonary carcinogenesis.

Key Words : Fhit, Immunohistochemistry, Non-small cell lung carcinoma, p53

Deletions of the short arm of chromosome 3 are considered critical events in the pathogenesis of lung cancer.¹ The *FHIT* (fragile histidine triad) gene is located within the FRA3B region, which is highly susceptible to breaks and deletions induced by carcinogens such as those in tobacco smoke.² It is a tumor suppressor gene at 3p14.2 and a common target of deletions in primary human cancers of epithelial origin, including those of the lung, head and neck, stomach, cervix, breast, and kidney. The analysis of *FHIT* RNA expression has shown *FHIT* alterations in these tumor types to be correlated with allelic deletions within the *FHIT* gene.¹ Although the biological function of the Fhit protein is still unknown, it is conceivable that *FHIT* plays a role in cell proliferation and/or apoptotic pathways.¹ There are several reports on the correlations between abnormalities of the *FHIT* gene and clinicopathological features in lung cancers,^{1,3} but the correlations are still unclarified, in particular with prognosis. Therefore, it is important to examine *FHIT* alterations in a large number of primary

lung carcinomas to assess the role of Fhit expression in multistage lung carcinogenesis. It is very difficult to understand the status of the *FHIT* gene locus in cancer cells by molecular analysis, because the *FHIT* gene spans over 1Mb in the FRA3B common fragile site at 3p14.⁴ Immunohistochemical detection has the advantage of detecting protein loss regardless of the underlying mechanism, and thus represents an efficient method of identifying functional protein inactivation.⁵ Two large studies on 608 and 106 non-small cell lung cancer (NSCLC) cases reported that there was an association of Fhit protein expression with the histotype, smoking history, and a shortened survival in stage I NSCLC by immunohistochemistry (IHC).^{1,4} A direct link between exposure to carcinogens contained in tobacco smoke and genetic abnormalities involved in bronchial carcinogenesis is now emerging. Mutations of *p53*, *K-ras*, and *FHIT* genes are among the most frequent gene alterations detected to date in lung cancer caused by smoking.⁶ In the group of subjects who had never smoked, loss of heterozygosity

(LOH) at 3p14.2 and *p53* mutations were present and constantly associated,⁶ but another study reported that Fhit expression was not correlated with the abnormal immunohistochemical expression of *p53* in NSCLC⁵ and in gastric cancers.⁷

In this report, we studied Fhit expression in a spectrum of non-small cell lung cancers by IHC and correlated the findings with clinicopathologic features including histologic types and smoking history. In addition, we investigated the relationship between loss of Fhit expression and *p53* overexpression in the pathogenesis of lung cancer.

MATERIALS AND METHODS

Patients

From January 1997 to April 2000, 43 primary lung tumors and 2 bronchoscopic biopsy specimens were obtained from 44 patients with NSCLC, who underwent curative resections and bronchoscopy at DanKook University Hospital. Among 43 resected tumors, two tumors of different histologic types were separately obtained from both lungs of one patient. We reviewed patient hospital records to find out the clinicopathologic variables such as age, sex, smoking history, TNM stage, and histologic grades and types of tumors. All cases were staged according to the Tumor-Node-Metastasis Classification of the AJCC staging system.⁸ All NSCLCs were histologically classified according to the "Histological Typing of Lung Tumors" by WHO (1981).⁹

Immunohistochemical staining for Fhit and *p53* proteins

Paraffin-embedded, 4- μ m-thick tissue sections from all 43 primary lung tumors and 2 bronchial mucosal biopsies were stained for the Fhit and *p53* proteins using, respectively, a primary rabbit anti-glutathione S transferase-Fhit polyclonal antibody (ZR44, Zymed) and a primary mouse anti-Human-*p53* monoclonal antibody (DO7, Novocastra, U.S.A.). Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed through graded alcohols. The sections were microwaved in a 10 mM citrate buffer at 90°C for 10 min and were treated with a 3% H₂O₂-PBS solution to reduce

endogenous peroxidase activity. They were then incubated with normal bovine serum to reduce nonspecific antibody binding and were subsequently subjected to the primary antibody reactions. The antibodies for Fhit and *p53* proteins were reacted with the sections at room temperature for one hour at the 1:200 and 1:100 dilution, respectively. Detection of the immunoreactive staining was carried out by the avidin-biotin-peroxidase complex method using the LSAB kit (DAKO, U.S.A.). The sections were subjected to a color reaction with 3,3-diaminobenzidine tetrahydrochloride containing 3% H₂O₂ in Tris buffer and were lightly counterstained with Mayer's hematoxylin.

The immunostained slides were interpreted according to the four-tiered scoring system by Tseng et al.,³ that incorporates both intensity of staining (absent, 0; low level, 1; moderate, 2; and strong, 3) and the percentage of cells stained (<5%, 1; 5-50%, 2; and >50%, 3). A composite score of 0-9 for the Fhit and *p53* protein levels was obtained by multiplying the intensity and extent scores for each tissue section. Composite scores ≤ 3 were defined as a marked reduction or absence of the proteins, whereas composite scores > 3 were considered positive for the protein expressions.

Statistical Analysis

Fisher's exact test was used to analyze the association between two categorical variables. $p < 0.05$ was considered to be statistically significant.

RESULTS

Clinicopathologic Features

The main clinicopathological features of 45 cases of NSCLC are listed in Table 1. The patients ranged from 31 to 83 years old, and the mean age was 59.9 years. The tumors were histologically classified as 25 squamous cell carcinomas (SCC), 15 adenocarcinomas (ADC), 3 large cell carcinomas (LCC), 1 bronchioloalveolar carcinoma (BAC), 1 adenosquamous carcinoma (ASC). The tumors were also graded as 6 cases of grade 1 (well differentiated), 23 cases of grade 2 (moderately differentiated), and 9 cases of grade 3 (poorly differentiated). The patients were staged as 15 cases of stage 1, 9 cases of stage 2, and 19 cases of stage 3. T

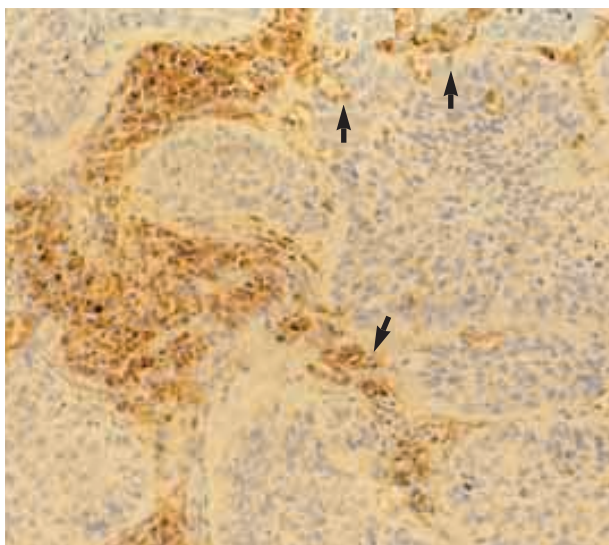


Fig. 1. A squamous cell carcinoma shows Fhit-negative immunostaining in all tumor cells and Fhit-positive immunoreactivity in the adjacent inflammatory cells and some small entrapped alveoli (arrows).

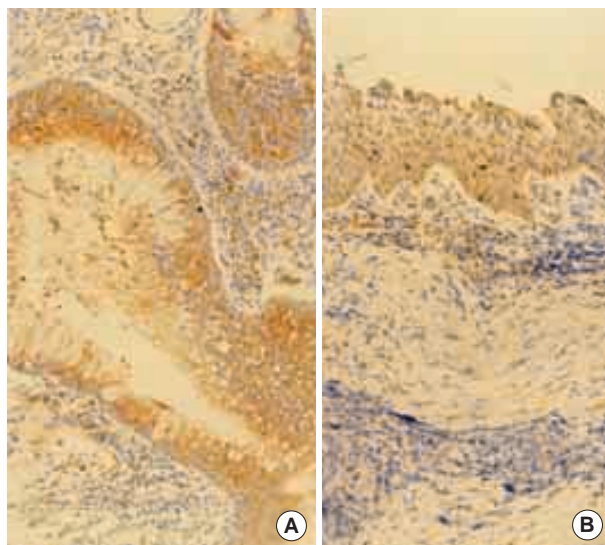


Fig. 2. Normal bronchial ductal epithelia (A) and bronchoscopic biopsy showing squamous metaplasia (B) show diffuse Fhit-positive immunoreactivity.

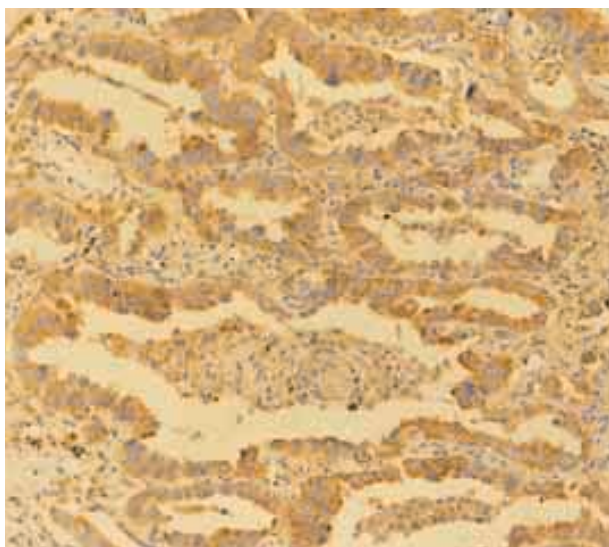


Fig. 3. A well-differentiated adenocarcinoma is diffusely immunoreactive for Fhit protein.

pathological stage revealed pT1 in 8 cases, pT2 in 18 cases, pT3 in 13 cases, and pT4 in 4 cases.

Loss of Fhit protein expression in NSCLC

Table 1 lists the frequency of Fhit reactivity in the tumor samples. Of 45 tumors analyzed, 35 (77.8%) revealed neg-

ative reactivity for Fhit protein expression. The percentage of negative cases was higher in squamous cell carcinomas compared with adenocarcinoma (92 versus 60%; $p < 0.05$). In squamous cell carcinomas, the loss of Fhit protein expression was restricted mainly to the tumor cells, and thus, contrasted with the positively stained entrapped alveolar linings and inflammatory cells in the adjacent areas (Fig. 1). The Fhit protein was clearly detectable by immunohistochemistry in the normal bronchial epithelia within the resected tumor sections and in the squamous metaplasia of the bronchoscopic biopsy specimens (Fig. 2). Adenocarcinoma specimens tended to reveal positive Fhit immunostaining more frequently and intensely than the squamous cell carcinomas (Fig. 3). These findings demonstrate that the loss of Fhit protein expression is a very frequent change in lung tumors, particularly in the squamous type of lung carcinoma. No association was found between Fhit-negative immunostaining and most clinicopathological variables such as age, sex, stage, pathological T stage, and histologic grade (Table 1).

Loss of Fhit expression and smoking habits

When Fhit expression was compared with smoking habits (pack/day \times year), the frequency of Fhit-negative tumors in smokers was significantly higher than in tumors

Table 1. Frequency of Fhit reactivity in the tumor according to the main clinicopathologic features

Clinicopathologic features	No. of cases	Fhit reactivity		p value
		Negative (%)	Positive (%)	
Age				
Mean age	59.9 yrs			
Age range	31-83 yrs			NS
Age≤60 yrs	21	17 (80%)	4 (20%)	
Age>60yrs	23	17 (74%)	6 (26%)	
Sex				
Male	36	31 (86%)	5 (14%)	NS
Female	8	5 (62.5%)	3 (37.5%)	
Stage				
1	15	10 (67%)	5 (33%)	NS
2-3	28	23 (82%)	5 (18%)	
pT				
1-2	26	19 (73%)	7 (27%)	NS
3-4	17	14 (82%)	3 (18%)	
Grade				
1	6	3 (50%)	3 (50%)	NS
2,3	32	27 (84%)	5 (16%)	
Histologic type				
SCC	25	23 (92%)	2 (8%)	<0.05
ADC	15	9 (60%)	6 (40%)	
LCC	3	3 (100%)	0	
BAC	1	0	1	
ASC	1	0	1	
Total	45	35 (77.8%)	10 (22.2%)	

SCC: squamous cell carcinoma, ADC: adenocarcinoma, LCC: large cell carcinoma, BAC: bronchioloalveolar carcinoma, ASC: adenosquamous carcinoma.

Table 2. Smoking habits and Fhit reactivity

Smoking Hx.	No. of cases	Fhit reactivity		p value
		Negative (%)	Positive (%)	
All cases				
Smokers	31	28 (90%)	3 (10%)	<0.05
Nonsmokers	8	4 (50%)	4 (50%)	
Data UA	5			
Adenocarcinoma only				
Smokers	11	8 (73%)	3 (27%)	NS
Nonsmokers	3	1 (33%)	2 (67%)	
Data UA	1			

Hx.: history, UA: unavailable.

Table 3. Smoking habits and p53 overexpression

Smoking Hx.	No. of cases	p53 overexpression		p value
		Present	Absent	
Smokers	31	24 (77%)	7 (23%)	NS
Nonsmokers	8	7 (88%)	1 (12%)	
Data UA	5			

Hx.: history, UA: unavailable.

Table 4. Correlation between Fhit and p53 gene alterations

p53 overexpression	No. of cases	Fhit reactivity		% Fhit negative	p value
		Negative	Positive		
Positive	34	28	6	82	NS
Negative	11	7	4	63	

from nonsmokers (90% versus 50%; $p < 0.05$) among all NSCLC cases (Table 2). When the patients with adenocarcinoma were studied separately, the correlation between lack of Fhit expression and smoking was not observed (73 versus 33%) (Table 2).

p53 overexpression and smoking habits

In contrast to the relationship between Fhit protein loss and smoking habits, p53 overexpression was not significantly different between the smoking and nonsmoking groups (77% versus 88%; Table 3).

Correlation of Fhit protein expression with p53 protein overexpression

The loss of the Fhit protein in the tumor samples (77.8%) was similar in overall frequency to p53 protein overexpression (75.5%). Fhit and p53 alterations were found to be independent events, because the difference in the frequency of Fhit-negative cases between the groups of p53-positive and -negative tumors was not statistically significant (82% versus 63%; Table 4).

DISCUSSION

Our observation of the loss of Fhit protein expression in a substantial percentage (77.8%) of primary NSCLCs suggests that *FHIT* alterations play an important role in the growth control of bronchial cells. This was particularly true for squamous cell carcinomas in which 92% of the cases showed loss of Fhit expression and the frequency was significantly higher than in adenocarcinomas ($p < 0.05$). Our data add to the growing body of evidence that loss of Fhit expression is distinctively more frequent in lung cancers of squamous differentiation. Two other immunohistochemical studies also showed that squamous carcinomas were significantly more likely to be Fhit-negative than

adenocarcinomas.¹⁴ In addition, it was previously shown that LOH at the *FHIT* locus is less common in adenocarcinomas than in squamous carcinomas.^{10,11} The particularly frequent loss of *FHIT* expression in the squamous type lung cancers seems to provide additional evidence that different histologic types may be associated with particular molecular alterations.⁵ In general, the squamous type of lung cancer is more closely related with smoking history than the adenocarcinoma. We, and Sozzi et al,¹ found a significant correlation between smoking habits and loss of *FHIT* reactivity in all tumors with smoking histories. This finding is consistent with a previous observation of a higher rate of LOH at the *FHIT* locus in the lung tumors of smokers than in those of nonsmokers.¹² This suggests that *FHIT* plays a specific role as a sensor of smoke-related carcinogenic damage leading to the loss of *FHIT* function. The evaluation of expression of the Fhit protein directly in tumor and bronchial sections simplifies the study of *FHIT* involvement in tumors of the lung as well as other tumor types where abnormalities of the *FHIT* gene have been reported.^{7,13,14} Recently, it was also reported that alterations in the *FHIT* locus detected by DNA and/or reverse transcription-polymerase chain reaction analysis correlate with the loss of Fhit protein expression in tumors.⁴ Additionally, immunocytochemistry seems to be more sensitive in the detection of Fhit alterations, as compared to the analysis of genomic and transcriptional changes, because several tumor specimens showed a lack of the Fhit protein in the absence of detected gene alteration.² This could be particularly true in lung tumors where the large amount of infiltrating stromal and inflammatory tissue can limit the sensitivity of molecular analysis performed on grossly dissected tumor specimens.² Thus, the lack of Fhit protein expression in lung tumors implicates *FHIT* in lung cancer pathogenesis, and our study demonstrated the role of Fhit protein in the development of lung cancers by revealing a high frequency (77.8%) of Fhit protein loss in NSCLCs. It was previously suggested that the genetic changes at the *FHIT* locus may be correlated with *p53* mutations,^{6,11} while other investigators found no statistically significant correlations between *FHIT* and *p53* abnormalities in NSCLCs.¹⁵ Geradts and associates (2000) also found that loss of Fhit expression was not associated with *p53* abnormalities in exon 5-8 by polymerase chain reaction single strand conformation polymorphism or *p53* accumulation by IHC.⁵ In our series, loss of Fhit expression was not cor-

related with *p53* protein overexpression by IHC. Likewise, in the immunohistochemical study by Sozzi and coworkers, Fhit-negative cases occurred at comparable frequencies in *p53*-positive and *p53*-negative NSCLCs.¹ In regard to relationships with other genes such as *K-ras*, *RB*, and *p16*, it has been reported that lack of Fhit staining was inversely correlated with codon 12 mutations in *K-ras* and not associated with *RB* and *p16* abnormalities.⁵ Also, a few studies demonstrated no significant association between *FHIT* LOH and *K-ras* abnormalities.^{6,15} The lack of association between the loss of Fhit expression and multiple other molecular abnormalities emphasizes the independent nature of disrupting the Fhit pathway in lung carcinogenesis. We found that the loss of Fhit protein expression was not significantly associated with a number of clinicopathologic parameters including age, gender, histologic grade, pT, and TNM stage. This is in agreement with the findings of other investigators, who also failed to detect a prognostic effect of aberrant Fhit expression in the resected NSCLCs.^{1,15}

In conclusion, these results indicate that the loss of Fhit expression plays an important role in the smoking-related carcinogenesis of NSCLC, especially in squamous cell carcinomas, and the *FHIT* gene could represent an eligible target for gene therapy approaches. In addition, *FHIT* and *p53* alterations are thought to be independent events in the development of NSCLCs.

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