Altered Expression of Fhit in Carcinoma and Precarcinomatous Lesions of the Esophagus

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Abstract

The FHIT gene, located at chromosome 3p14.2, is a tumor suppressor gene often involved in tumors resulting from exposure to environmental carcinogens. We studied 46 pairs of esophageal primary tumors and corresponding normal squamous mucosa specimens by molecular genetic and immunohistochemical methods to investigate the role of the FHIT gene in esophageal carcinoma. In addition, we studied several different types of lesions, such as carcinoma in situ or dysplasia by immunohistochemistry. Loss of heterozygosity at or around the FHIT gene was observed in 35 (76%) primary tumors. Immunohistochemical detection of Fhit protein in the primary tumors demonstrated that 14 (30%) were positive and 32 (70%) were negative. We observed concordance between loss of Fhit protein and loss of heterozygosity and between loss of Fhit protein and RNA abnormalities. Because the FHIT/FRA3B locus is susceptible to damage by environmental carcinogens, we investigated the correlation between Fhit expression and smoking or alcohol habits. In this relatively small study, the patients who were both heavy users of tobacco and alcohol showed a significantly higher frequency of loss of Fhit expression than those who were light users. Noncarcinomatous squamous epithelium showed positive Fhit reactivity in most cases; however, five showed negative Fhit reactivity. Interestingly, all of these five patients had habits of heavy use of tobacco and alcohol. Eight of 12 carcinomas in situ, 2 of 4 severe dysplasias, 4 of 8 moderate dysplasias, and 3 of 9 mild dysplastic lesions showed negative Fhit reactivity. These findings indicated that loss of Fhit expression may be an early event in the development of human esophageal carcinoma and may occur even in normal-appearing squamous epithelium in some patients heavily exposed to environmental carcinogens.

Introduction

Deletions of the short arm of chromosome 3 have been reported in a variety of common tumors, including esophageal carcinoma. Efforts to identify the altered gene(s) on chromosome 3p have led to the discovery of the FHIT gene located at chromosome region 3p14.2 (1). The FHIT gene is more than 1 Mb in size, encodes a 1.1-kb cDNA with 10 small exons, and encodes a cytoplasmic M_1_ protein with diadenosine triphosphate (Ap3A) hydrolase activity (1, 2). Previous studies have shown that the FHIT gene is inactivated by deletions in carcinoma cell lines and primary tumors of the lung, head and neck, breast, stomach, colon, and several others (3), and sequencing of nearly 900 kb of the gene has led to precise definition of biallelic deletions within the FHIT gene in representative cancer-derived cell lines (4, 5).

There are a few reports concerning the role of the FHIT gene in esophageal carcinoma (6, 7). We first studied FHIT gene alteration in 10 cases of human esophageal squamous cell carcinomas and demonstrated that five cases showed aberrant transcripts determined by RT-PCR (1). We suggested that these aberrant RT-PCR products might result from the loss of genomic regions encompassing FHIT exons. A study of Michael et al. (6) demonstrated that alterations of FHIT transcripts were observed in 14 of 15 adenocarcinomas of the esophagus and FHIT homozygous deletions in at least 25% of the cases. On the other hand, Zou et al. (7) studied 13 esophageal carcinomas by RT-PCR and demonstrated that 1 of 13 expressed no detectable FHIT transcript, and the other 12 showed normal-sized transcripts. We have now expanded the number of cases examined and studied the correlation among DNA and RNA alterations and loss of FHIT expression to determine the role of FHIT in human esophageal carcinomas.

Recently, loss of FHIT expression was reported to be associated with exposure to environmental carcinogens (8). Because smoking and alcohol consumption are risk factors for the development of esophageal carcinoma (9), we also investigated the correlation between tobacco or alcohol habits and FHIT status in esophageal carcinomas. In addition, because previous studies have reported loss of Fhit expression in precarcinomatous lesions of the lung (10), we investigated whether this loss of Fhit expression occurs in precancerous lesions of the esophagus. Here we have shown that loss of the Fhit protein occurs in the majority of esophageal carcinomas, and that loss of Fhit expression occurs also in the precarcinomatous conditions, such as dysplasia or carcinoma in situ. Very interestingly, loss of Fhit expression was also detected in the surrounding normal-appearing squamous epithelium of some patients with high exposure to carcinogens.

Materials and Methods

Clinical Cases. Forty-six Japanese patients with esophageal carcinomas were included in this study. The patients underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University, Beppu, and Saitama Cancer Center Hospital, Saitama, Japan. The patients included 42 males and 4 females. Ages ranged from 48 to 74 years (mean, 60 years). Forty-four cases had squamous cell carcinomas, and two had small cell carcinomas, histologically. None of the 46 showed adenocarcinoma. Five were stage I, four were stage II, and 35 were stage III, according to the Tumor-Node-Metastasis classification.

The data on smoking and alcohol habits were obtained for 39 patients. Most patients were cigarette smokers and/or alcohol drinkers. Only two were nonsmokers, and one a nondrinker. The extent of smoking was classified by Brinkman index: heavy, >900; and light, <900, according to the median number per year. The grade of alcohol drinking was classified by alcohol index

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2 To whom requests for reprints should be addressed, at Kimmel Cancer Center, 233 South 10th Street, Philadelphia, PA 19107.
3 The abbreviations used are: FHIT, fragile histidine triad; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity.
grams of alcohol/day × year): heavy, ≥2300; and light, <2300, according to the median number per year.

**Tissue Samples and DNA/RNA Extraction.** Tumor tissues and the corresponding normal tissues were obtained for each patient. The tissue samples were excised and immediately stored at −80°C. DNA and RNA were extracted from each sample according to methods described previously (11, 12).

**LOH Study.** The loci examined were D3S1234, D3S1295, D3S1300, D3S1312, and D3S1313. D3S1300 and D3S1312 are located in intron 5 of the **FHIT** gene locus (5). D3S1295 and D3S1313 are telomeric to the **FHIT** gene, whereas D3S1312 is centromeric at 3p14.2. These microsatellite sequences were obtained from the Genome Database (GDB) and primers were labeled by 5'-fluorescein phosphoramidite or 5-tetrachlorofluorescein phosphoramidite for microsatellite loci, as described by Ishii et al. (13). Cases were judged to exhibit LOH when an allele peak signal from tumor DNA was reduced by 50% compared with the normal counterpart, as described (13). The optimal conditions for amplification of those microsatellite loci have been described elsewhere (1, 10, 14).

The oligonucleotides for generating PCR products from **FHIT** exons for agarose gel analysis were designed from the previous studies (1, 15). Primers iex5F/R, iex6F/R, iex7F/R, iex8F/R, and iex9F/R were used for exons 5, 6, 7, 8, and 9, respectively. PCR amplifications were carried out in 30 μl of final volume with 100 ng of genomic DNA template, 10 pmol of primers, 10 mM

<p>| Table 1. <strong>FHIT</strong> gene in primary esophageal cancers |
|----------------------------------|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>D3S1234</th>
<th>D3S1313</th>
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<th>EX7</th>
<th>EX6</th>
<th>D3S1234</th>
<th>D3S1300</th>
<th>EX5</th>
<th>D3S1312</th>
<th>RT-PCR</th>
<th>Protein</th>
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| E01 | | | | | | | | | | | 5-
| E02 | | | | | | | | | | | 5-
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| E04 | | | | | | | | | | | 5-
| E05 | | | | | | | | | | | 5-
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| E07 | | | | | | | | | | | 5-
| E08 | | | | | | | | | | | 5-
| E09 | | | | | | | | | | | 5-
| E10 | | | | | | | | | | | 5-
| E11 | | | | | | | | | | | 5-
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| E13 | | | | | | | | | | | 5-
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| E17 | | | | | | | | | | | 5-
| E18 | | | | | | | | | | | 5-
| E19 | | | | | | | | | | | 5-
| E20 | | | | | | | | | | | 5-
| E21 | | | | | | | | | | | 5-
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| E23 | | | | | | | | | | | 5-
| E24 | | | | | | | | | | | 5-
| E25 | | | | | | | | | | | 5-
| E26 | | | | | | | | | | | 5-
| E27 | | | | | | | | | | | 5-
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| E34 | | | | | | | | | | | 5-
| E35 | | | | | | | | | | | 5-
| E36 | | | | | | | | | | | 5-
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| E38 | | | | | | | | | | | 5-
| E39 | | | | | | | | | | | 5-
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| E44 | | | | | | | | | | | 5-
| E45 | | | | | | | | | | | 5-
| E46 | | | | | | | | | | | 5-
| E47 | | | | | | | | | | | 5-
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| E57 | | | | | | | | | | | 5-
| E58 | | | | | | | | | | | 5-
| E59 | | | | | | | | | | | 5-
| E60 | | | | | | | | | | | 5-
| E61 | | | | | | | | | | | 5-

(, microsatellite retained; 5, microsatellite LOH; 5, microsatellite deleted; 5, FHIT exon retained; 5, FHIT exon deleted. 5, nonvaluable; N, normal sized transcripts; A, aberrant transcripts; Ni, not informative)

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TRIS-HCl (pH 8.3), 50 mM KCl, 0.1 mg/ml gelatin, 1.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate, and 0.5 unit of Taq polymerase (ABI). The amplifications were performed in a Perkin-Elmer Cetus thermal cycler for 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s. Amplified exon fragments from normal and tumor DNA were run on agarose gels to identify homozygously deleted exons.

**RT-PCR and DNA.** cDNA was synthesized from 2 μg of total RNA. RT-PCR was performed as described previously (12). DNA bands corresponding to the normal and abnormal size FHIT transcripts were excised from the gel, purified using the Quick Gel extraction kit (Qiagen, Inc., Valencia, CA), and sequenced on the Applied Biosystems model 373A and 377 DNA sequencers (Applied Biosystem, Inc., Foster City, CA).

**Immunostaining.** Expression of the Fhit protein in paraffin-embedded tumor sections was examined by immunohistochemical staining as described previously (16–18) with a polyclonal serum specific for human Fhit (15, 16). All primary tumors and the corresponding noncancerous squamous mucosa were studied. Detailed pathological examination of the 46 resected esophageal samples showed that there were several kinds of premalignant lesions in some cases in addition to the primary malignant tumors. We thus selected several samples that were studied by immunohistochemistry; these included 12 lesions of carcinoma in situ, 4 lesions of severe dysplasia, 8 lesions of moderate dysplasia, and 9 lesions of mild dysplasia. The histological classification was performed according to the criteria of Enterline and Thompson (19).

The primary antibody was omitted and replaced by PBS (Life Technologies, Inc., Grand Island, NY) in the negative controls. The adjacent normal squamous epithelium was used as an internal positive control. When the normal squamous epithelium stained negative, the esophageal glands located in the lamina propria mucosa served as a positive control. Immunohistochemical staining was classified in the following two groups: negative, no staining was present or positive staining was detected in <10% of the cells; and positive, >10% of the cells stained positive. Two independent readers (M. M. and H. I.) were involved in the assessment of expression.

**Clinical and Pathological Comparison between FHIT-positive and -negative Cases.** Fhit-positive and -negative cases were compared with respect to age, sex, location of the tumor, histological differentiation, depth of tumor invasion, lymphatic permeation, vascular vessel invasion, lymph node metastasis, stage of the disease, or prognosis.

**Statistical Analysis.** The Fisher’s exact test with a two-tailed P was used in analysis of statistical significance of correlation between clinicopathological variables and FHIT expression. Survival curves were calculated by Kaplan-Meier estimate, using log-rank testing for the assessment of statistical significance.

**Results**

The results of DNA analysis, mRNA expression determined by RT-PCR, and immunostaining are summarized in Tables 1 and 2. The correlation between FHIT and LOH or mRNA expression status is summarized in Table 2. The correlation between FHIT reactivity and smoking or alcohol consumption is summarized in Table 3. The grade of alcohol drinking is classified by alcohol index (grams of alcohol/day × year): heavy, ≥900 per year, NS, not significant. Light or no alcohol.

**LOH Study.** The DNA analysis demonstrated that LOH and/or homozygous loss of markers was observed in 35 cases (76%; Table 1). Seven cases showed homozygous deletion of exons of the FHIT gene as follows: cases 32, 36, 40, 46, and 56 lost from exon 5 to exon 9, which includes the entire protein coding region of the FHIT gene; case 45 lost exons 7 and 5; and case 53 lost exon 5. On the other hand, 11 cases did not show LOH and apparently retained all FHIT exons (Table 1).

**mRNA Expression.** As shown in Table 2, the RT-PCR study demonstrated that no FHIT transcripts were amplified from 8 tumors, and aberrant transcripts plus normal-sized FHIT RT-PCR products were amplified in 17 tumors. One case showed only aberrant transcripts. Sequence analysis of aberrant transcripts showed the absence of coding and noncoding exons and nucleotide insertions. The remaining 20 tumors showed only normal-sized transcripts. All 46 samples from normal tissue showed normal-sized product. Very interestingly, however, aberrant products were also recognized in normal tissue samples from six patients who were both heavy smokers and drinkers, as mentioned below. We checked the remaining samples of these six normal tissues by histological examination and made sure that the samples contained only normal (noncanceromatous) tissue. The aberrant products were studied by sequence analysis, and the results showed the absence of coding and noncoding exons and nucleotide insertions. Five of these six showed negative Fhit staining in the normal (noncanceromatous) tissues, as mentioned below.

**Immunostaining.** The summary of the immunostaining study and photographs of representative cases are shown in Table 2 and Fig. 1, respectively. The study demonstrated that 14 cases showed positive staining and 32 showed negative staining in carcinoma cells. As shown in Table 2, in 35 cases with LOH, 5 showed positive immunostaining and 30 showed negative immunostaining for Fhit. On the other hand, among 11 cases with no LOH, 9 showed positive immunostaining and 2 showed negative immunostaining for Fhit. There was a significant correlation between LOH status and immunostaining status (P < 0.01). Then we compared the results of RT-PCR with those of immunostaining. Many cases (15 of 18) with aberrant RT-PCR products showed negative immunostaining. On the other hand, more than half of the cases (11 of 20) with normal-sized RT-PCR products showed negative immunostaining.

Normal (noncanceromatous) squamous epithelium showed positive Fhit reactivity in most cases; however, five cases showed negative Fhit reactivity, not only in the primary tumor but also in the normal squamous epithelium. Interestingly, all of these five patients had habits of heavy tobacco and alcohol consumption, suggesting an association between Fhit protein loss and environmental carcinogen exposure. Eight of 12 carcinomas in situ, 2 of 4 severe dysplasias, 4 of 8 moderate dysplasias, and 3 of 9 mild dysplastic lesions showed negative Fhit reactivity (Fig. 1).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Correlation among Fhit reactivity, LOH, and aberrant mRNA expression</th>
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<tbody>
<tr>
<td>LOH</td>
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<tr>
<td>Present</td>
<td>Positive</td>
</tr>
<tr>
<td>Absent</td>
<td>Normal</td>
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<tr>
<td>mRNA expression</td>
<td>11</td>
</tr>
</tbody>
</table>

† This is a comparison between the normal expression group and the other two groups.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Smoking, alcohol habits, and Fhit reactivity</th>
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</thead>
<tbody>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Heavy</td>
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</tr>
<tr>
<td>Light or no</td>
<td>9</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
</tr>
<tr>
<td>Heavy</td>
<td>4</td>
</tr>
<tr>
<td>Light or no</td>
<td>10</td>
</tr>
<tr>
<td>Both smoking and alcohol</td>
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<td>Heavy</td>
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</tr>
<tr>
<td>Light</td>
<td>8</td>
</tr>
</tbody>
</table>

† Five of these 11 patients show negative Fhit reactivity in neither carcinomatous tissue nor adjacent noncanceromatous epithelium.
Loss of Fhit Expression and Tobacco and Alcohol Consumption. Twenty patients were heavy smokers, 17 were light smokers, and 2 were nonsmokers. Twenty patients were heavy drinkers, 18 were light drinkers, and only 1 was a nondrinker. The correlation between Fhit reactivity and smoking or alcohol habits is summarized in Table 3. Eleven of 12 patients with both heavy smoking and drinking habits showed negative Fhit reactivity. On the other hand, 8 of 13 patients with either light or nonsmoking and nondrinking habits showed positive Fhit reactivity.

Fig. 1. Immunohistochemical staining for Fhit in esophageal diseases. A, esophageal carcinoma covered by normal squamous epithelium (×40). Note negative (thick arrow) or very weak (thin arrow) staining in the carcinoma tissue and positive staining in normal squamous epithelium. B, H&E staining in the same area of A. C, a lesion of severe dysplasia indicated by a line (×80). Note negative staining in severe dysplasia and positive staining in normal squamous epithelium. D, H&E staining in the same area of C. E, a case of negative staining in normal squamous epithelium (×120). This patient was a heavy smoker and drinker, and the primary tumor also stained negative. Note positive staining only in the ductal epithelium of esophageal glands. F, H&E staining in the same area of E. G, a case of positive staining in primary squamous carcinoma (×140). Note that carcinoma cells stained positive for Fhit. H, H&E staining in the same area of G.
showed positive Fhit reactivity. There was a significant difference between the two. Five of 12 patients with both heavy smoking and drinking habits showed no Fhit reactivity in the carcinomatous tissue and normal tissue.

**Clinical and Pathological Features of Fhit-positive and -negative Cases.** There was no significant correlation between Fhit-positive (n = 14) and -negative (n = 32) cases with any of the factors studied. The 5-year survival rate was 36% in Fhit-positive cases and 32% in Fhit-negative cases.

**Discussion**

Investigation of primary esophageal cancers showed that loss of Fhit expression occurred in 70% of the tumors. These results are very similar to those of non-small cell lung carcinomas (73%; Ref. 10), gastric carcinomas (67%; Ref. 17), and pancreatic carcinomas (62%; Ref. 20). Loss of Fhit expression was associated with LOH (genomic

**FHIT** alteration) and altered Fhit transcripts, as shown in Table 2. Similar findings were observed in other carcinomas such as those of lung (10) or stomach (17).

Several investigators have reported that loss of Fhit expression might be associated with carcinogen-induced damage. For example, in lung carcinomas, the loss of Fhit expression or LOH in the Fhit locus was higher in tumors or bronchial lesions of smokers than those of nonsmokers (10, 21). With respect to esophageal carcinoma, smoking and drinking are two major risk factors (9). Thus, we studied the relationship between Fhit expression and smoking and/or drinking habits (Table 3). Because most of the patients included in this study were smokers and/or drinkers, we divided the patients into two groups (heavy and light) according to the median amounts of tobacco or alcohol use. The results showed that there was no significant difference in loss of Fhit expression between heavy and light smokers and between heavy and light drinkers, although the heavy drinkers had a tendency toward a higher frequency of loss of Fhit expression (P = 0.07). If these two habits were combined, the patients with both heavy habits showed a significantly higher frequency of loss of Fhit expression than those with light habits of consumption of both (P < 0.01; Table 3). The findings support the hypothesis that loss of Fhit expression is associated with exposure to environmental carcinogens, although larger studies, including more cases with light or no habits of consumption, will be needed to clarify the relationship between carcinogen exposure and esophageal cancer.

Loss of Fhit expression was already detectable in precarcinomatous lesions of the tongue; Sozzi et al. (10) reported that loss of Fhit expression was recognized in 100% of carcinomas in situ (n = 25) and 85% of dysplasia (n = 20). Our present study also demonstrated that loss of Fhit expression was seen in 67% of carcinomas in situ (n = 12) and 43% of dysplasia (n = 21). Although the frequency of loss of Fhit expression was lower in lesions of esophagus than lung, these results plus the observation of loss of Fhit expression in normal squamous epithelium of patients with both heavy smoking and drinking habits support the proposal that loss of Fhit is an early event in the pathogenesis of esophageal carcinoma.

There are two possibilities concerning the reduction of Fhit expression in esophageal tumors. One is that it occurs quite early in the development of carcinoma, as mentioned above and seen in this study of esophageal carcinoma and other studies of lung carcinoma (10, 21). The other is that it may accompany progression toward a more aggressive form of disease. In fact, loss of Fhit has been reported to correlate with more aggressive disease in bladder (22) or breast (14) carcinomas. Our study of esophageal carcinoma demonstrated that there were no significant clinicopathological differences between the cases with positive and negative Fhit reactivity with respect to depth of tumor invasion, lymphatic or vascular vessel invasion, or lymph node metastasis. Additional studies are necessary to clarify the relative importance of Fhit alterations in the development or progression of carcinomas.

Stable Fhit-transduced clones expressing exogenous wild-type Fhit showed reduced colony-forming efficiency in vitro and loss of ability to form tumors in nude mice (18). Recently, the mechanisms involved in suppression by Fhit has been examined by Sard et al. (23), who reported that growth-inhibitory effects in Fhit expressing cells may be related to apoptosis and cell cycle arrest in a p53-independent manner. Because esophageal carcinoma is one of the most aggressive diseases and shows poor prognosis (24), a new therapy such as Fhit gene therapy could represent a desirable breakthrough.

**References**


Altered Expression of Fhit in Carcinoma and Precarcinomatous Lesions of the Esophagus

Masaki Mori, Koshi Mimori, Takeshi Shiraishi, et al.

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