Loss of Fragile Histidine Triad Expression in Colorectal Carcinomas and Premalignant Lesions

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Abstract

Abnormal expression of the fragile histidine triad (FHIT) candidate tumor suppressor gene has been observed in a variety of human tumors, but little is known about its expression during colorectal tumorigenesis. Sections of 70 aberrant crypt foci (ACF), 55 adenomas, 84 primary colorectal carcinomas, and 13 metastatic lesions were evaluated immunohistochemically for FHIT expression. All normal colonic epithelium showed a strong expression of FHIT; 44% of carcinosmas showed a marked loss or absence of FHIT expression. The proportion of carcinomas with reduced expression showed an increasing trend (a) with decreasing differentiation and (b) in tumors with metastasis (62%) compared with tumors without metastases (38%). The proportion of metastatic lesions (12 of 13) with reduced expression of FHIT was even greater. Although only a small proportion of ACF and adenomas showed a reduction of FHIT expression, the reduced expression of FHIT was strongly associated with the degree of dysplasia in both ACF (P = 0.0002) and adenomas (P = 0.0085). The findings of reduced expression of FHIT in a small proportion of colonic precancerous lesions and in increased proportions of primary and metastatic colorectal cancers suggest that FHIT plays a role in the development and progression of some colon carcinomas.

Introduction

The FHIT gene has been cloned recently and mapped to chromosomal region 3p14.2(1). It spans not only the t(3:8)(p14.2;q24) translocation breakpoint found in familial renal cell carcinoma but also the most common human fragile site, FRA3B(2). Abnormalities in the FHIT gene and/or its expression have been identified in a variety of human cancer cell lines and tumor tissues including lung (3, 4), breast (5), head and neck (6), esophageal (7, 8), gastric (9), pancreatic (10), renal (11), and cervical (12) cancer. Abrerrant protein expression and allelic deletion of FHIT in lung cancer are associated with smoking history and prognosis (3, 4). The finding of decreased expression of FHIT in 93% of precancerous lesions of the lung suggested that this gene might be used as an intermediate biomarker for the early diagnosis and/or prevention of lung cancer (4). A few studies have evaluated the FHIT gene in colorectal cancer, but some of the data are conflicting. Ohta et al. (12) reported three of eight primary colon tumors with aberrat FHIT transcripts, and Kastury et al. (13) found nearly 50% of colorectal cancers with loss of heterozygosity. In contrast, Thiagalingam et al. (14) suggested that “FHIT is inactivated by an unusual mechanism or that it plays a role in relatively few colorectal tumors” because they found (a) no somatic point mutations detected by sequence analysis of the complete coding regions, and (b) 29 of 31 colorectal cancers exhibited normal mRNA transcripts.

Materials and Methods

Specimens. Paraffin-embedded sections of 55 colorectal adenomas and 53 carcinomas were obtained from the Academic Department of Pathology, St Mark’s Hospital (London, United Kingdom). All of the remaining tissues were obtained from the Western division of the Cooperative Human Tissue Network of the National Cancer Institute located at Case Western Reserve University. These tissues included 31 additional carcinomas and 70 ACF; 58 ACF were from 33 patients with sporadic colon cancer, and 12 ACF were from 4 patients with FAP. The cancers were staged by Dukes’ criteria. When distant metastases were present, we classified the tumor as stage D. Of the 31 patients with carcinomas, 12 were Dukes’ stage C or D. Metastatic lesions including 3 from liver and 10 from lymph nodes were obtained from surgically resected tissue from these patients.

One of the adenomas was from a patient with a history of FAP, the remaining 54 adenomas were from patients with an average age of 58.9 ± 11.4 years (28 males and 26 females). Three carcinomas were from FAP patients; the remaining 81 carcinomas were from patients with an average age of 66.3 ± 13.8 years (44 males and 37 females). The sporadic colon cancer patients with FHIT used in this study had an average age of 70.9 ± 12.9 years (17 males and 16 females).

Immunohistochemical Analysis. Formalin-fixed paraffin-embedded sections were cut at 5 μm and placed on 3-aminopropyltriethoxysilane (Sigma, St Louis, MO)-coated slides or Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). One section was stained with H&E and used for histological classification, and the others were used for immunostaining.

Slides were deparaffinized in xylene twice for 7 min, rehydrated through graded ethanol to distilled water, and heated in 0.01 m citrate buffer (pH 6.0) in a pressure cooker for 3 min after reaching full pressure for freshly cut slides or slides kept at 4°C. The time was increased to 7 min for slides kept for several years at room temperature. The sections were incubated for 15 min in a blocking solution containing 10% normal goat serum in PBS [0.01 m phosphate (pH 7.4), 0.137 m NaCl] and then incubated for 1 h at 37°C in a humidified chamber with rabbit polyclonal anti-glutathione S-transferase-Fhit fusion protein antibodies (Zymed Laboratories Inc., South San Francisco, CA) diluted 1:200 in blocking solution. The sections were rinsed in PBS and incubated for 30 min with biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in blocking solution. To block endogenous peroxidase activity, the slides were

Photomicrographs of FHIT protein expression in colon carcinomas have been published previously (1), but there is no detailed investigation of FHIT protein expression during colorectal tumorigenesis. Colon cancer, like lung cancer, is thought to be induced by carcinogens; therefore, it is imperative to determine whether FHIT plays a role in this second most common cause of cancer deaths in the United States (15). In this study of 84 colorectal carcinomas, 55 adenomas, and 70 ACF, we now report that FHIT protein expression was altered in a high proportion of colorectal carcinomas and metastatic lesions. Although only a small proportion of adenomas and ACF had reduced expression of FHIT, this loss of FHIT expression was strongly correlated with dysplasia.

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1 The abbreviations used are: ACF, aberrant crypt foci; FAP, familial adenomatous polyposis.
immersed in 3% hydrogen peroxide in 30% methanol for 10 min. After washing in distilled water, the sections were then incubated for 30 min in streptavidin-biotinylated horseradish peroxidase complex (Amersham, Arlington Heights, IL) diluted 1:100 in blocking solution. 3,3'-Diaminobenzidine (Sigma) was used as the chromogen. Slides were counterstained for 3 min with 0.1% methyl green and covered with 50% Clearium/50% xylene (Surgipath Medical Industries, Inc., Richmond, IL). Normal colonic epithelium was used as a positive control for every lesion, whereas the primary antibody was replaced by normal rabbit serum IgG with a similar dilution for a negative control.

Fig. 1. Expression of Fhit protein by immunohistochemical staining of human colonic specimens embedded in paraffin. A, normal colonic epithelium with strong expression of Fhit protein from the bottom to the top of the crypts, ×50; B, colon carcinoma with strong cytoplasmic expression of Fhit protein, ×200; C, colon carcinoma with weak expression of Fhit protein, ×120; D, metastatic colon cancer lacking Fhit expression from the same patient whose primary tumor is illustrated in B, ×120; E, H&E-stained section of an adenoma with varying degrees of dysplasia; an asterisk (*) marks the same gland here and in F, ×50; F, heterogeneous expression of Fhit in the same adenoma as in E, ×50; G, low-power view of a H&E-stained section of an ACF marked with yellow ink and arrows at the top, ×50; H, low-power view of the same ACF marked with arrows showing a marked reduction of Fhit expression compared with Fhit expression in the normal glands adjacent and below it, ×50; I, higher magnification of the same ACF as in G marked with arrows, ×120; J, higher magnification of the same ACF marked with arrows showing a marked reduction of Fhit expression in the ACF, ×120.
The following trends were observed: the proportion of tumors with reduced expression increased with decreasing differentiation ($\chi^2 = 5.76$, df = 1, $P = 0.016$); the proportion of tumors with reduced expression increased in tumors with metastases (Dukes’ stage C + D) compared with tumors without metastases (Dukes’ stage A + B; $\chi^2 = 3.68$, df = 1, $P = 0.055$). There was a lack of association of differentiation with Fhit expression ($P = 0.1204$, Fisher’s exact test); and of Dukes’ stage with Fhit expression ($P = 0.4512$, Fisher’s exact test).

**Evaluation of Score.** Both the extent and intensity of immunopositivity were considered when scoring Fhit protein expression. The extent of positivity was scored as follows: 0, <5%; 1, >5–25%; 2, >25–50%; 3, >50–75%; and 4, >75% of the colonic epithelial cells in the respective lesions. The intensity was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, as strong as normal mucosa. The final score was obtained by multiplying the extent of positivity and intensity scores, producing a range from 0 to 12 (16). Scores 9–12 were defined as preserved or strong staining pattern because there was little difference compared with normal mucosa, scores 0–4 were defined as markedly reduced or lost expression, and scores 6–8 were defined as intermediate staining pattern.

**Statistical Analyses.** Fisher’s exact test (two-sided), McNemar’s $\chi^2$ test with continuity correction, and the Cochran-Armitage test for trends in proliferation were used to adjust for the multiple testing.

**Results**

**Fhit Expression in Normal Mucosa and Carcinomas.** All normal colonic epithelium showed strong cytoplasmic expression of Fhit protein from the basal cells to the luminal differentiated cells (Fig. 1A); these served as an internal control. Some stromal cells, such as macrophages, also stained with Fhit antibodies. Fhit protein expression was retained (Fig. 1B) in 33 of 84 (39%) carcinomas, was intermediate or heterogeneous in 14 carcinomas (17%), and was markedly reduced or absent (Fig. 1C) in 37 of 84 (44%) carcinomas (Table 1). The proportion of carcinomas with reduced expression of Fhit protein showed an increasing trend ($\chi^2 = 5.76$, df = 1, $P = 0.016$) from 2 of 10 (20%) well-differentiated cancers to 30 of 68 (44%) moderately differentiated cancers to 5 of 6 (83%) poorly differentiated cancers (Table 1). A similar trend was observed with Dukes’ stage 24 of 63 (38%) Dukes’ stage A and B cancers had reduced expression of Fhit compared with 13 of 21 (62%) Dukes’ stage C and D cancers ($\chi^2 = 3.68$, df = 1, $P = 0.055$). However, no overall associations were found between Fhit expression and either the degree of differentiation or Dukes’ stage (Table 1).

There was a marked increase in the proportion of metastatic lesions (12 of 13 lesions; 92%) with reduced expression of Fhit protein (Fig. 1D) compared with that observed in 37 of 84 (44%) primary colorectal cancers ($\chi^2 = 8.642$, df = 1, $P = 0.0033$). Of the 12 patients with metastatic lesions analyzed, the only one who retained strong Fhit expression in both the primary and metastatic lesions was a patient with FAP. Two other patients had strong Fhit expression in their primary tumors (Fig. 1B) with reduced expression in their metastases (Fig. 1D), and nine patients had reduced Fhit expression in both their primary and metastatic lesions, i.e., there was not a significant difference in Fhit expression between primary and metastatic lesions from the same patients ($P = 0.495$, McNemar’s $\chi^2$ test).

**Fhit Expression in Premalignant Lesions: Adenomas and ACF.** For the 55 adenomas, there was an association ($P = 0.0085$, Fisher’s exact test) between the loss of Fhit expression and the degree of dysplasia (Table 2). All 19 adenomas with mild dysplasia retained the strong expression of Fhit displayed by normal mucosa; the higher grades of dysplasia showed weaker expression of Fhit (Table 2, Fig. 1, E and F). A smaller proportion (6 of 36; 17%) of adenomas with moderate or severe dysplasia showed reduced expression of Fhit ($\chi^2 = 7.0691$, df = 1, $P = 0.0078$) than that (44%) observed in carcinomas (Tables 1 and 2).

For the 70 ACF, there was a strong association ($P = 0.0002$, Fisher’s exact test) between the loss of Fhit expression and the degree of dysplasia (Table 3). All but 1 of 50 ACF with atypia or mild dysplasia displayed strong Fhit expression; i.e., only a small proportion of ACF had reduced expression of Fhit (Fig. 1, B and F). All three ACF with severe dysplasia from sporadic colon cancer patients exhibited reduced expression of Fhit (Table 3). It is interesting to note that all 12 ACF from four different patients with FAP retained strong Fhit expression, regardless of their histology. There was no difference ($P > 0.83$, $\chi^2$ test) in the expression of Fhit in adenomas with moderate or severe dysplasia as compared with ACF with moderate or severe dysplasia (Tables 2 and 3).

**Discussion**

In this study, we have demonstrated that 44% of colorectal cancers have markedly reduced expression of Fhit protein. A similar reduction of Fhit protein expression has been reported in other human tumors such as lung (4), cervical (12), renal (11), pancreatic (10), head and neck (6), and breast (5) carcinomas. The frequent loss of Fhit protein expression has been reported in other human tumors (8). Additional evidence that suggests that Fhit is important in colon tumorigenesis. A trend of increased proportions of colorectal cancers

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<td>Total</td>
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<sup>a</sup> WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
expressed reduced levels of Fhit (a) with decreasing degrees of differentiation, (b) with more advanced stages (Dukes’ stage C and D) compared with less advanced stages (Dukes’ stage A and B) of primary tumors, and (c) in metastatic lesions compared with primary tumors. These data suggest that the loss of Fhit expression is associated with the progression of colorectal cancer and may play a role in the tumorigenic process. Similar losses of FHT function have been associated with stage, grade, and poor prognosis in lung cancer (3) and advanced disease in breast cancer (5).

Alterations of the FHIT gene and/or its expression have also been reported in preneoplastic lesions of the lung (4), esophagus (7), and cervix (12). Aberrant mRNA transcripts have been reported in preneoplastic lesions of the colon (18). ACF are putative precancerous lesions that are reported in premalignant lesions of the lung (4), esophagus (7), and cervix and advanced disease in breast cancer (5).

Associated with stage, grade, and poor prognosis in lung cancer (3)
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in a significant proportion of colorectal cancers and in an even higher proportion of metastatic lesions. Although the proportion of adenomas and ACF with reduced expression of Fhit was small, this reduced expression showed a strong association with dysplasia. These results suggest that FHIT plays a role in the development and progression of colorectal cancer from the premalignant stage through metastasis.

References

In summary, the expression of Fhit was markedly reduced or absent in a significant proportion of colorectal cancers and in an even higher proportion of metastatic lesions. Although the proportion of adenomas and ACF with reduced expression of Fhit was small, this reduced expression showed a strong association with dysplasia. These results suggest that FHIT plays a role in the development and progression of colorectal cancer from the premalignant stage through metastasis.
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