Loss of FHT Expression in Gastric Carcinoma

Raffaele Baffa, Maria Luisa Veronese, Roberto Santoro, Bernadette Mandes, Juan P. Palazzo, Massimo Rugge, Eugenio Santoro, Carlo M. Croce, and Kay Huebner

Kimmel Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania 19107; [R. B., M. L.V., B. M., J. P. P., C. M. C., K. H.]; 2nd Department of Oncologic Surgery, Istituto Regina Elena, 00161 Rome, Italy [R. S., E. S.]; and Department of Pathology, University of Padova, 35100 Padova, Italy [M. R.]

ABSTRACT

Loss of heterozygosity involving the short arm of chromosome 3 has been reported in gastric and other human tumors. We have cloned and mapped a candidate tumor suppressor gene, FHT (fractogenic histidine triad), to this chromosomal region (3p14.2). To investigate the role of FHT gene alterations in the development of gastric carcinoma, we examined 8 gastric carcinoma-derived cell lines and 32 primary adenocarcinoma samples by Southern blot analysis. We also analyzed the integrity of FHT transcripts by reverse transcription-PCR. The occurrence of alterations in the FHT gene and its transcript correlated with the absence of Fhit protein expression by immunoblot analysis in the cancer cell lines. Four of eight cell lines showed deletion or rearrangement within the FHT gene, together with the absence of the wild-type transcript and the Fhit protein. Among the primary gastric carcinomas, rearrangement of the FHT gene and/or aberrant reverse transcription-PCR products were detected in 17 of 32 (53%) tumors, and 20 of 30 (67%) samples exhibited an absence of Fhit protein expression. Gastric cancer is thought to develop from carcinogenic exposure, possibly explaining the high frequency of abnormalities in the FHT gene, a fragile locus exhibiting susceptibility to carcinogen-induced alterations. The consequent absence or reduction of Fhit protein expression is consistent with the proposal that the FHT gene is a preferential target of environmental carcinogens and that FHT inactivation plays a role in the development of gastric cancer.

INTRODUCTION

Gastric cancer was considered the leading cause of cancer-related death in the United States in 1930. Over the last 60 years, its incidence has decreased dramatically; in 1995, gastric cancer ranked fourteenth among the major types of cancer in the United States, with 22,800 patients diagnosed each year (1). Despite this declining rate in the United States, stomach cancer remains the second most frequent cause of cancer-related death in the world (2), with mortality rates higher than 50 per 100,000/year in regions such as China, Eastern Europe, and South America (3). Carcinoma of the stomach is associated with specific risk factors, the most important of which are Helicobacter pylori infection, alcohol consumption, tobacco smoking, increased salt and nitrate intake, and decreased antioxidant vitamin intake (4-7).

In 1965, Lauren (8) classified carcinomas of the stomach into two histological types, intestinal (well differentiated) and diffuse (poorly differentiated). The histogenesis of the intestinal and diffuse types differ, with the intestinal type being the result of a well-defined multistep process from normal mucosa to adenocarcinoma, whereas the intermediate steps leading to the diffuse type remain unknown (9, 10). A number of investigators have used molecular genetic analyses to demonstrate that differences in the natural history of intestinal and diffuse types of gastric carcinoma are consequences of different genetic events (reviewed in Ref. 11).

Allelotypes of solid tumors determined by screening for LOH at polymorphic loci are effective in identifying the chromosomal locations of potential tumor suppressor genes, with chromosome regions 1q, 3p, 3q, 5q, 6q, 7q, 11p, 11q, 12q, 13q, 17p, and 18q being frequently involved in gastric cancer (12-20). We suggested previously (21) that a locus on chromosome 3p14.2 plays a role in the development of gastric cancer because of the high frequency of LOH (46%) in primary stomach tumors, and because homozygous deletions at this region were observed in two stomach tumor-derived cell lines. We subsequently identified and characterized the FHT gene at 3p14.2 and showed it to be abnormally expressed in tumors of the gastrointestinal tract (22). Since then, numerous reports have suggested the FHT gene as the specific target of 3p14.2 alterations in several types of human cancers (23-28).

The FHT gene encompassing the FRA3B fragile region encodes a protein of ~16,800 with diadenosine triphosphate hydrolase activity dependent on the conserved histidine triad encoded by exon 8 (29). A papillomavirus insertion site (30), plasmid integration sites (31), and cancer-specific translocations (21, 32) have been mapped within the FHT gene. In addition, we observed a suppression of tumorigenicity in cancer-derived cell lines, including two cell lines derived from gastric carcinoma, after restoring Fhit protein expression (33).

Previous studies had suggested that the FHT gene may be involved in gastric cancer (26, 34). We now show that loss of the Fhit protein occurs in a majority of gastric cancers of both the diffuse and the intestinal type, suggesting that loss of FHT function is a critical step in gastric tumorigenesis.

MATERIALS AND METHODS

Cell Lines and Tissues. Seven gastric cancer-derived cell lines (Ags, Hs 746T, Kato III, NCI-N87, RF-1, RF-48, and SNU-16) were obtained from the American Type Culture Collection. The Mgc 80-3 cell line was provided by Si-Chun Ming (Temple University, Philadelphia, PA). As summarized in Table 1, four cell lines were derived from intestinal stomach adenocarcinomas, and three cell lines were derived from diffuse tumors (35-38); no clear data on histotype were reported for the Hs 746T cells.

Specimens from 32 tumors were obtained from resected stomach cancers at Istituto Regina Elena (Rome, Italy). Samples were taken immediately after resection and divided into two fragments. One portion was snap-frozen in liquid nitrogen and used for molecular studies, and the other was processed for routine histopathological analysis. No microdissection was performed in any of the samples. However, the ratio of tumor: normal cells was evaluated by resection and divided into two fragments. One portion was snap-frozen in liquid nitrogen and used for molecular studies, and the other was processed for routine histopathological analysis. No microdissection was performed in any of the samples. However, the ratio of tumor: normal cells was evaluated by analyzing frozen sections of all of the specimens, and the percentage of tumor cells was determined to be between 60 and 75%. The tumors were classified histologically according to Lauren's classification (8) and staged according to the tumor-node-metastasis (TNM) classification of malignant tumors as defined by the International Union Against Cancer. Matched normal gastric mucosa samples were available from 26 patients.

RNA and cDNA Sequence Analysis. Total RNA was extracted from cell lines and tissues using the RNA-STAT kit (TelTest, Inc., Friendswood, TX).

Received 5/22/98; accepted 8/10/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by National Cancer Institute Outstanding Investigator Grant CA 39860 (C. M. C.), Grant PO1 CA 21124, National Cancer Institute Cancer Center Grant CA56336 (to the Kimmel Cancer Center), and a gift from R. R. M. Carpenter III and M. K. Carpenter.

2 To whom requests for reprints should be addressed, at Kimmel Cancer Institute, Jefferson Medical College, BLSB, Room 1035, 233 South 10th Street, Philadelphia, PA 19107. Phone: (215) 503-4643; Fax: (215) 923-3528.

1 The abbreviations used are: LOH, loss of heterozygosity; RT, reverse transcription; TBST, Tris-buffered saline, 0.1% Tween; GST, glutathione S-transferase.

4708
and cDNA was synthesized from 2 μg of RNA. RT was carried out in a 22-μl volume with 300 units of Superscript II (Life Technologies, Inc., Gaithersburg, MD), 500 ng/μl oligo(dT), and 50 ng/μl random hexamers. The reaction was incubated at 42°C for 50 min and boiled for 5 min. The final reaction was diluted with distilled water to 30 μl, and 1 μl of cDNA was used for PCR in a mixture of abnormal FHIT transcripts that lacked one or more exons of the FHIT gene or lacked exons with the insertion of intronic sequences, as described previously (23).

Southern Blot Analysis. Genomic DNA (~7 μg) from primary tumors and cell lines was digested with BamHI, and the products were separated on 0.8% agarose gels, blotted onto nylon membranes, and hybridized as described previously (39). FHIT cDNA (spanning exons 1–9); cosmids B4, C6 (spanning FHIT exon 5), and C63 (a portion of intron 5); and 05#l probes (spanning FHIT exons 6 and 7; Ref. 22) were labeled with the Prime-It II kit (Stratagene, La Jolla, CA). Preannealing and hybridization reactions were performed as described previously (23).

LOH Analysis. DNA from normal/tumor pairs was extracted by standard techniques (39) and amplified by PCR using primers for loci within or flanking the FHIT gene. The following six loci were analyzed: (a) D3S1312 (centromeric to FHIT exon 1); (b) D3S2620 in FHIT intron 4; (c) D3S300 and D3S4103 in FHIT intron 5; (d) D3S134 (between exons 7 and 9); and (e) D3S3131 (telomeric to FHIT exon 10). PCR amplification reactions were performed using 100 ng of template in a 25-μl volume using 1 unit of Taq polymerase (TaKaRa, PanVera Corp., Madison, WI); 50 mM each of dATP, dTTP, and dGTP; 5 mM dCTP; and 10 μCi of [α-32P]dCTP. Amplifications were carried out for 19–23 cycles at the appropriate temperature for each primer. Electrophoresis and assessment of LOH were performed as described previously (18).

Western Blot Analysis. Cultures of gastric cancer cell lines were lysed for the detection of Fhit protein expression by immunoblot analysis. Total cellular protein (100 μg) was mixed with 5× PAGE loading buffer and denatured for 3–5 min at 90°C before loading on a 12% SDS-PAGE gel. After gel electrophoresis and electrotransfer, the nitrocellulose membrane was blocked with 5% milk-TBST buffer for 1 h at room temperature, washed with TBST, and incubated with purified polyclonal anti-GST-Fhit serum (1:2,000 dilution; Ref. 32) for 1 h at room temperature. After repeating the washing steps described above, antirabbit immunoglobulin labeled with horseradish peroxidase (Amersham, Arlington Heights, IL; diluted 1:40,000 in TBST buffer) was added to the membrane and incubated for an additional hour at room temperature, and the washing steps described above were repeated. Enhanced chemiluminescence detection reagents (Amersham) were used to detect the Fhit protein.

Immunohistochemistry. Dewaxed 5-μm formalin-fixed paraffin-embedded serial sections obtained from the same block used for the histological diagnosis were immersed in citrate buffer (0.01 M sodium citrate (pH 6) and boiled for 10 min to enhance antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 30 min. The slides were incubated with the above-described anti-GST-Fhit antibody (1:4000 dilution) overnight at room temperature. The primary antibody was replaced by PBS in the negative controls. The avidin-biotin complex was used according to the manufacturer’s directions (ABC kit; Vector Laboratories, Inc., Burlingame, CA). Antibody localization was detected using diaminobenzidine as a chromogen substrate. Finally, sections were washed with distilled water and weakly counterstained with Harris’ hematoxylin. Immunoreactivity was scored as present or absent. The specificity of the anti-GST-Fhit serum for detection of the Fhit protein was demonstrated previously (32, 33, 40).
covering intronic and exonic regions revealed homozygous deletion in the Ags, Kato III, and NCI-N87 cell lines (Fig. 2, B and C) and rearranged restriction fragments in some primary tumor DNAs (Fig. 3, B-D). When the filters were hybridized with cosmid B4, rearranged bands were detected in five cases (Ro12T, Ro18T, Ro27T, Ro29T, and Ro34T; Fig. 3B). Case Ro12T showed a deletion when hybridized with cosmid c76, and case Ro27 showed a deletion when hybridized with cosmid 05#1 (Fig. 3D). A total of 11 of 32 (34%) primary gastric
cancer specimens showed rearrangement or deletion within the *FHIT* gene.

**LOH Analysis.** Polymorphic loci spanning chromosome band 3p14.2 were analyzed by PCR amplification to determine the prevalence of allelic deletion at the *FHIT* locus in gastric cancer. Allelic loss was evaluated in 60 paired normal and tumor specimens at loci D3S4260, D3S1300, D3S4103, and D3S1234 internal to the *FHIT* gene and at loci centromeric (D3S1312 at 3p14.2) and telomeric (D3S1313 at 3p14.3) to the *FHIT* gene. Of the 60 specimens, which were all informative for at least one marker, 26 (43%) showed allelic imbalance at 3p14.2. A summary of RT-PCR, Southern blot, and LOH analyses for 32 of the 60 specimens for which both DNA and RNA were available is presented in Table 2.

**Fhit Protein in Stomach Carcinoma.** Immunoblot experiments in the eight gastric cancer cell lines showed that the cell lines with a homozygous deletion at the *FHIT* locus (Ags, Kato III, and NCI-N87) did not express Fhit protein, nor did Mgc 80-3 cells with a rearrangement of *FHIT* exon(s) and an absence of normal-sized *FHIT* transcript (Fig. 4). Conversely, the remaining four cell lines (Hs 746T, RF-1, RF-48, and SNU-16) expressed abundant Fhit protein in concordance with the apparent absence of *FHIT* gene alteration in these cells, as shown in Fig. 4 and summarized in Table 1. Thus, there was a direct correlation in stomach cancer cell lines between *FHIT* gene alterations and an absence of expression of the Fhit protein.

The same polyclonal antibody was used for immunodetection of Fhit protein on formalin-fixed paraffin-embedded sections from 30 primary adenocarcinomas of the stomach. Positive Fhit immunostaining was observed in normal gastric glands as shown in Fig. 5A. In 20 of 30 tumors, Fhit immunoreactivity was undetected, suggesting that in 67% of stomach cancers, Fhit protein was absent or greatly reduced; examples are shown in Fig. 5, and protein expression results are summarized in Fig. 1. A and B, and Table 2.

**Correlations among the Parameters Assessed.** Fig. 1. A and B, illustrates the relationship between Fhit protein expression and the spectrum of RT-PCR products observed in the primary and cultured stomach cancers, and these data are compared with the results of tests for DNA alterations in Table 2. Cases in Table 2 are divided into groups A–D, depending on whether *FHIT* gene alterations correlated with the absence of the Fhit protein. Cases Ro21 and Ro35 were not evaluated for protein expression and are thus excluded from the groups. Groups A and B include 16 cases (53%) that show a direct correlation between *FHIT* genetic lesions and the effect on Fhit protein. Group A shows the summary for 10 Fhit-negative cancers that showed genomic alterations and/or RT-PCR abnormalities; group B included the six Fhit-positive cancers that showed no evidence of *FHIT* genomic lesions, except for case Ro36, which may have lost one *FHIT* allele.

Group C included 10 cases that were Fhit negative but showed no evidence of *FHIT* genomic alterations. It is possible that the *FHIT* locus has been inactivated by deletion in these cases, and that the lack of LOH and the presence of normal RT-PCR products reflect the presence of nonneoplastic cells in the tumor samples. However, the results for this group resemble those we have reported for clear cell renal carcinomas: an absence of protein with no evidence of genomic
FHIT IN STOMACH CANCER

Fig. 3. Southern blot analysis of the FHIT gene in primary tumors. A. hybridization of BamHI-digested DNAs from primary gastric carcinomas with a FHIT cDNA probe. Asterisks: missing exons in cases Ro12T, Ro13T, Ro19T, and Ro21T. Case Ro32 is an example of a comparison between tumor DNA (Ro32T) and its normal counterpart (Ro32N). B. hybridization of the same DNAs with cosmid B4. Asterisks: rearranged restriction fragments in cases Ro12T, Ro18T, Ro27T, Ro29T, and Ro34T. C. hybridization with cosmid c63. Asterisk: a rearranged restriction fragment in Ro35T. D. hybridization with cosmid 0501. Asterisk: the absence of a restriction fragment in Ro27T.

lesions (41). We hypothesize that FHIT mRNA expression is very low in these tumors, as it is in many lung cancer, cervical cancer, and other cancer cell lines (22, 40, 42), possibly due to the methylation of a CpG island at the 5' end of the gene.

Group D contained four Fhit-positive tumors (13%) that showed Fhit expression in apparent discordance with the presence of FHIT genetic alterations. These cancers showed evidence of FHIT lesions by Southern blot or RT-PCR assay but still expressed Fhit protein. These tumors may be examples of cancers in which only one FHIT allele has been inactivated by deletion or rearrangement, whereas the other FHIT locus is still intact and encodes Fhit protein.

No significant correlation was noted between the absence of Fhit expression and the histotype or the stage of the tumors. Nevertheless, combining the results of the cell lines and primary adenocarcinomas, we noted a trend toward a higher frequency of Fhit immunonegativity in intestinal-type carcinoma (76%) compared with the diffuse-type (50%) gastric adenocarcinoma.

DISCUSSION

Among human solid tumors, adenocarcinoma of the stomach represents a particularly interesting model for studying interactions between differentiation, transformation, and genetic alterations in epithelial cells. Lauren's (8) original classification of gastric carcinoma into intestinal and diffuse types is based on the growth pattern and morphology of the neoplastic glands (8). The histogenesis of the intestinal and diffuse types are believed to be different, with the intestinal type resulting from a long-lasting multistep process that progresses from superficial gastritis, chronic gastritis, atrophy, intestinal metaplasia, and dysplasia to advanced carcinoma, whereas the intermediate steps leading to the diffuse type remain unknown. In the past decade, several reports suggested different molecular pathways for the development of the two histotypes (43, 44). Our finding of a high incidence (46%) of LOH at 3p14.2 in gastric cancer and the definition of a small region of homozygous deletion (60 kb) in a gastric carcinoma cell line (Kato III) contributed to the positional cloning of the 1-Mb FHIT gene that includes fragile region FRA3B (21), the most highly inducible fragile site in the genome. In the same study, abnormal FHIT expression in five of nine stomach cancers was detected by RT-PCR analysis. Conflicting reports have since appeared concerning the possible role of FHIT inactivation in gastric carcinogenesis (26, 34). We have previously correlated DNA abnormalities at the FHIT locus with aberrant RT-PCR products and altered FHIT
expression in tumor cell lines (32). In addition, using specific polyclonal antibodies against the Fhit protein, we and others observed concordance between RNA abnormalities and the reduction and/or absence of Fhit protein in cell lines and primary tumors of the lung (45) and of the uterine cervix (40). In this study, we used RT-PCR, Southern blot analysis, LOH, and protein studies to analyze six additional gastric cancer-derived cell lines and 32 primary adenocarcinomas of the stomach to further explore the role of the FHIT gene in gastric cancer development.

The absence of a wild-type FHIT transcript in four cell lines (Mgc 80-3, NCI-N87, Ags, and Kato III; Fig. 1A) is not an unexpected finding. As shown in Fig. 2, Southern analysis confirms the correlation between the presence of aberrant FHIT RT-PCR products and the rearrangements and/or homozygous deletion at the FHIT locus. Fig. 4 shows that there is no detectable Fhit protein in the cell lines with FHIT alterations. Thus, in the cell lines, there is a perfect correlation among FHIT DNA, RNA, and protein alterations.

Because of problems associated with contaminating normal cells in tumor samples, it is perhaps not surprising to find the absence of a normal FHIT transcript in only 2 of 32 primary tumors (Ro7T and Ro13T; Fig. 1B).

In summary, our results show that the FHIT gene is inactivated in the majority of gastric tumors and indicate that carcinogens such as N-nitroso compounds, free oxygen radicals, metabolites of tobacco (46, 47), and Helicobacter pylori infection could exert their carcinogenic potential on the gastric mucosa by affecting, directly or indirectly, the fragile region contained in the FHIT gene, thus causing breakage and/or deletion within this gene.

The observation that the Fhit protein is absent in 50% of gastric cancer cell lines and in 67% of primary gastric adenocarcinomas without regard to a specific histotype indicates that alterations of the FHIT gene can play a critical role in an early stage of gastric tumorigenesis. This also suggests that subsequent additional genetic changes involving other tumor suppressor genes or oncogenes may be responsible for tumor progression.

Because LOH at 3p14.2 has been reported at an early stage in oral and lung carcinogenesis (48, 49), our next step will be to assess Fhit protein expression in precancerous lesions of the stomach to determine the earliest morphological modifications of the gastric glands that show an absence of Fhit expression. Identification of these lesions may offer a new tool for the diagnosis of gastric cancer.

REFERENCES

Loss of *FHIT* Expression in Gastric Carcinoma

Raffaele Baffa, Maria Luisa Veronese, Roberto Santoro, et al.

*Cancer Res* 1998;58:4708-4714.

Updated version  Access the most recent version of this article at:  
[http://cancerres.aacrjournals.org/content/58/20/4708](http://cancerres.aacrjournals.org/content/58/20/4708)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.