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ORIGINAL ARTICLE

# Clinicopathologic Features and *FHIT* Gene Expression in Sporadic Colorectal Adenocarcinomas

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**Background:** The putative tumour suppressor gene *FHIT* (fragile histidine triad) spans the common fragile site FRA3B, which is highly susceptible to breaks and deletions induced by genotoxic agents. Tumours associated with exposure to carcinogens, such as colorectal adenocarcinomas, should be particularly susceptible to alterations in the *FHIT* gene. We studied the frequency of *FHIT* alterations and their correlations with clinicopathologic features in sporadic colon carcinomas. **Methods:** *FHIT* expression was investigated by reverse transcription polymerase chain reaction in 56 primary sporadic colorectal carcinomas. The same tumours and matched normal tissues were also investigated for loss of heterozygosity by using two markers located inside the *FHIT* gene. **Results:** Twenty-nine of 56 tumours (51.8%) expressed aberrant *FHIT* transcripts. Four tumours had absence or nearly undetectable levels of the normal-sized *FHIT* transcript. Sequencing analysis of the altered transcripts showed *FHIT* mRNA lacking one or more exons, more frequent deletions of exons 4-5-6 or 4-5-6-7-8. At the genomic level 46.4% (13 of 28) of the cases showed alterations involving *FHIT* locus. We did not find any correlation between *FHIT* gene alterations and clinicopathologic characteristics of the tumours. **Conclusions**: Since the *FHIT* gene is frequently altered, its role in the molecular pathogenesis of sporadic colon carcinoma deserves further investigation.

**Key words:** Aberrant transcripts; colorectal carcinomas; *FHIT* gene; tumour suppressor gene

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The short arm of chromosome 3 has been a hot topic for many investigators as a potential tumour suppressor gene site. Numerous studies have shown that chromosomes 3p allelic losses occur in many forms of carcinoma (1, 2), and experiments designed to introduce a normal chromosome 3 in tumour cells have consistently shown a reduction in tumorigenicity after chromosome transfer (3).

Recently, the *FHIT* (fragile histidine triad) gene has been cloned and mapped at 3p14.2 (4). A papillomavirus insertion site (5), plasmid integration sites (6), and carcinoma-specific translocations and deletions (7) have been located within the *FHIT* locus.

*FHIT* is the first gene found to span a common fragile site: FRA3B is the site on normal human chromosomes most sensitive to the formation of gaps or breaks when DNA replication is disturbed by aphidicolin or folate stress (8).

Abnormalities in the *FHIT* locus have been found in many established carcinoma cell lines, and the gene is abnormally transcribed in a broad range of primary tumours, suggesting its aetiologic role in human carcinogenesis (9–11). In particular, a very high frequency of *FHIT* alterations has been described in lung carcinomas (10). Marchetti et al. (12) reported that the frequency of loss of heterozygosity (LOH) at the *FHIT* locus is significantly higher in tumours from heavy-smoking subjects than in non-smoking patients, and *FHIT* exon deletions have been associated with the duration of smoking and with asbestos exposure (13). These observations suggest that environmental insults may result in *FHIT* alterations.

The intestinal tract, like the lungs, is directly exposed to environmental genotoxic agents. Otha et al. (4) found aberrant transcripts of the *FHIT* locus in oesophageal and stomach carcinomas and in three of eight primary colon adenocarcinomas analysed (4). To evaluate the involvement of the *FHIT* gene in the pathogenesis of colorectal carcinoma, we examined the expression of *FHIT* in 56 cases of colorectal carcinomas by reverse transcription (RT) of *FHIT* mRNA,

followed by nested-polymerase chain reaction (PCR). Deletions at the *FHIT* locus were also evaluated by using two intragenic polymorphic markers, D3S1300 (located within intron 5 of the *FHIT* gene locus, close to exon 5) and D3S1234 (located in intron 7). *FHIT* gene alterations were then correlated with tumour morphologic grade, clinical stage, and survival profile.

## Materials and Methods

*Patients and specimens*

Tissues were obtained from patients undergoing surgical resection for sporadic colon adenocarcinoma between 1994 and 1997. Tumour and normal tissue specimens were frozen immediately and stored in liquid nitrogen. The clinicopathologic features of each patient were reviewed and recorded. We obtained the follow-up information in only 54 of the 56 patients.

*RNA and DNA extraction*

Total RNA and DNA were extracted from frozen tumours and normal tissue using RNeasy and QIAamp tissue kits (QIAGEN, Hilden, Germany), in accordance with the manufacturer's specifications.

*RT-PCR*

cDNA was synthesized from 1 μg total RNA. Reverse transcription and the first-step PCR was performed in a total volume of 25 μl, using the SuperScript One-Step RT-PCR System (Life Technologies Italia S.r.L., San Giuliano Milanese, Italy). RNA, 1× reaction mix, 0.2 μM 5U2 and 3D2 primers (4), and 0.5 μl SuperScript II RT/Taq mix were first incubated at 30 °C for 30 min, denatured at 94 °C for 3 min, and amplified in a thermal cycler (GeneAmp (PCR System 9700, Perkin Elmer, Foster City, Calif., USA) for 25 cycles at 94 °C for 20 sec, 58 °C for 30 sec, 72 °C for 55 sec, and a final extension at 72 °C for 5 min.

*Nested PCR and sequencing*

One microlitre of the first-step PCR was diluted 50-fold in water, and 1 μl of the diluted reaction was used for the second-step PCR amplification, using nested primers 5U1 and 3D1 (4) for 25 cycles under the above conditions. PCR reactions were carried out in a 25-μl volume containing: 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers, and 1.25 units of Taq polymerase (Advanced Biotechnologies, UK). The PCR products were resolved in a 1.5% ethidium bromide-stained agarose gel in Tris/boric acid/ethylenediaminetetraacetic acid (EDTA) (TBE) buffer, and bands were cut from gels and purified using a QIA quick gel extraction kit (QIAGEN).

cDNA was sequenced by using various *FHIT*-specific, overlapping primers, a dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), and a DNA sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer), following the manufacturer's protocol.

*LOH analysis*

LOH analysis was done with a PCR-based approach with primers amplifying polymorphic microsatellite alleles at loci D3S1300 (within intron 5 of the *FHIT* gene locus, close to exon 5) and D3S1234 (located in intron 7). One of two primers from each marker was first end-labelled with $^{33}$P-adenosine triphosphate (ATP), 2000 Ci/mmol (NEN, Germany), and T4 DNA polynucleotide kinase (Pharmacia Biotech Italia, Cologno Monzese, Italy). PCR reactions were carried out in a 15-μl volume containing about 150 ng genomic DNA, 1 × PCR buffer, 2 mM $MgCl_2$ 0.25 mM dNTPs, 0.2 μM primers, 0.2 μl of the end-labelled primer, and 1.25 units of Taq polymerase (Advanced Biotechnologies). The two marker PCR conditions were the same: 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were separated on a 7% polyacrylamide–urea–formamide gel and visualized by autoradiography.

*Statistical analysis*

The following variables were assessed: age (<60 or ≥60), sex, tumour grade (G1 = well differentiated, G2 = moderately differentiated, G3 = poorly differentiated, mucinous), Dukes stage (A = tumours confined to the muscularis propria, B = tumours invading the muscularis propria without lymph node involvement, C = tumours with lymph node metastases, and D = widely metastatic disease). Tumour location was categorized into three groups: right, left, and rectum. Tumours involving the caecum up to the splenic flexure were designated 'right' colon tumours; the splenic flexure, left colon, and sigmoid colon were designated 'left' colon tumours, whereas the sigmoid junction and intra- and extraperitoneal rectum were indicated 'rectal' tumours. In one case it was not possible to evaluate the grade of the tumour sample; therefore, the total number of graded tumours is only 55.

The statistical analysis was carried out using the STATA statistical package (STATA, College Station, Tex., USA).

The association between alterations in the *FHIT* gene and its transcripts and clinicopathologic features was assessed by using Fisher exact tests and the chi-square test. Kaplan–Meier curves were used to estimate survival probability as a function of time, and patient relapse or survival differences were analysed with the log-rank test.

## Results

We analysed 56 tumours from patients with sporadic colorectal adenocarcinoma. Fifty-two of the 56 colon adenocarcinomas analysed expressed wild-type *FHIT* transcripts at a robust level; additional bands were seen in 29 of 56 (51.8%) tumours (Table I). Four tumours had absent or nearly undetectable levels of the normal-sized *FHIT* transcript.

Table I. Clinicohistopathologic characteristics of patients with sporadic colorectal adenocarcinoma and their association with *FHIT* gene alterations

| Variable | Overall ($n = 56$) | *FHIT* without aberrant transcripts, $n$ (%) | *FHIT* aberrant transcripts, $n$ (%) | Chi-square | $P$ value |
|---|---|---|---|---|---|
| Age (years) | 64 ± 1.2* | 62 ± 2.1* | 64 ± 1.8* | | |
| <60 | 14 | 5 (35.7) | 9 (64.3) | 1.168 | NS |
| >60 | 42 | 22 (52.4) | 20 (47.6) | | |
| Sex | | | | 0.840 | NS |
| Male | 36 | 19 (52.8) | 17 (47.2) | | |
| Female | 20 | 8 (40) | 12 (60) | 0.42 | NS |
| Dukes stage | | | | | |
| A | 5 | 2 (40) | 3 (60) | | |
| B | 20 | 9 (45) | 11 (55) | | |
| C | 20 | 10 (50) | 10 (50) | | |
| D | 11 | 6 (54.54) | 5 (45.45) | 5.51 | NS |
| Tumour grade | | | | | |
| G1 | 3 | 0 | 3 (100) | | |
| G2 | 39 | 18 (46.15) | 21 (53.85) | | |
| G3 | 8 | 5 (62.5) | 3 (37.5) | | |
| Mucinous | 5 | 4 (80) | 1 (20) | 0.428 | NS |
| Survival | | | | | |
| Yes | 42 | 20 (74.1) | 22 (81.5) | | |
| No | 12 | 7 (25.9) | 5 (18.5) | 0.7013 | NS |
| Relapse | | | | | |
| Yes | 21 | 12 (57.1) | 9 (42.9) | | |
| No | 33 | 15 (45.45) | 18 (54.54) | 2.449 | NS |
| Tumour location | | | | | |
| Right | 15 | 5 (33.3) | 10 (66.7) | | |
| Left | 19 | 9 (47.4) | 10 (52.6) | | |
| Rectum | 21 | 13 (62) | 8 (38) | | |

* Mean ± standard error; NS = not significant.

Examples of tumours with aberrant transcripts are shown in Fig. 1.

Sequencing analysis showed that additional bands were *FHIT* transcripts lacking one or more exons with junctions precisely at the splicing sites. The abnormal transcripts were more frequently deletions of exons 4-5-6 resulting in the junction of exons 3–7, deletions of exons 4-5-6-7-8 resulting in the junction of exons 3–9, and deletions of exons 5-6-7 resulting in the junction of exons 4–8. We found only one case of an insertion after exon 3 of a 97-base sequence; a BLAST search of the GeneBank database showed a significant homology (78%) of 29 bases of the inserted sequence with the varicella-zoster virus sequence.

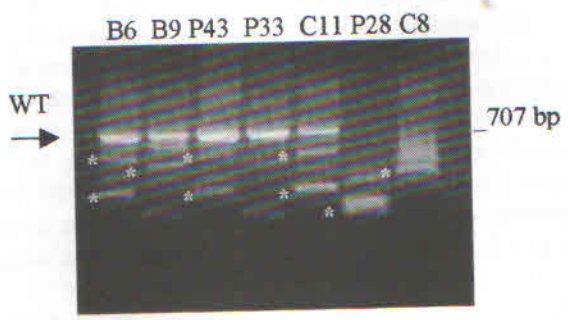


Fig. 1. Results of the reverse transcription polymerase chain reaction analysis of *FHIT* transcripts. The arrow shows the normal-size transcript (707 bp); asterisks indicate aberrant transcripts of the *FHIT* gene. Lack of normal *FHIT* expression is seen in tumours C8 and P28. WT = wild type.

Analysis of genomic DNA from 35 informative patients showed LOH at the *FHIT* locus in 7 of 35 (20%) cases. We also found six cases of microsatellite instability (shift or appearance of extra alleles in tumour DNA compared with normal DNA) (Table II). Examples of this analysis are shown in Fig. 2.

We did not find any statistically significant association between the presence of *FHIT* aberrant transcripts and age, sex, relapse, survival of the patient, Dukes stage, or grading and localization of the tumour (Table I). Our analysis showed no significant difference in survival or relapse for patients with or without *FHIT* gene alterations (LOH or aberrant transcripts).

## Discussion

In the present series of sporadic colorectal adenocarcinomas we found aberrant transcripts of the *FHIT* gene in 51.8% (29 of 56) of the tumours analysed, almost constantly accompanied by a normal-sized transcript. This frequency is comparable with those observed in colon tumours by Chen

Table II. Microsatellite analysis of sporadic colon adenocarcinomas

| Micro-satellite | Cases analysed | Informative cases | LOH (%) | Instability (%) |
|---|---|---|---|---|
| D3S1300 | 55 | 35 | 6/35 (17.4) | 1/35 (2.8) |
| D3S1234 | 55 | 35 | 1/35 (2.8) | 5/35 (14.3) |



... mRNA,

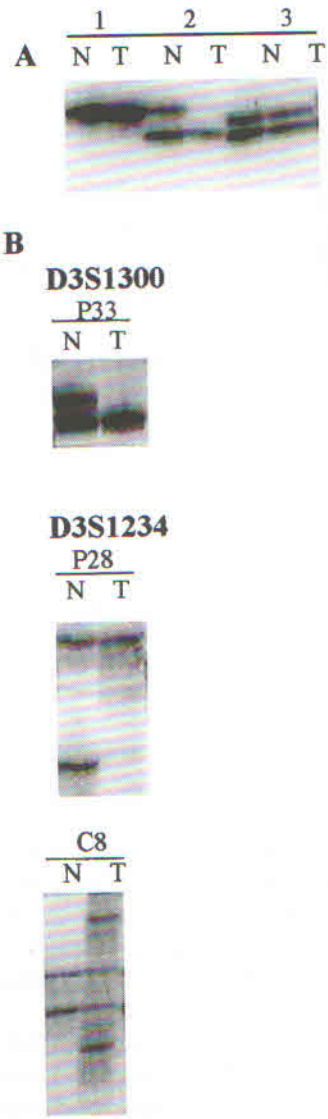


Fig. 2. Examples of allelic deletion analysis of primary colorectal adenocarcinomas. N = normal tissue DNA; T = tumour DNA. A. Case 1 was uninformative, case 2 showed loss of heterozygosity (LOH), and case 3 retained heterozygosity. B. Examples of LOH and instability at microsatellite loci D3S1300 and D3S1234.

et al. (14), using a similar experimental approach. Sequence analysis of the abnormal transcripts showed that they were generated by loss of internal exons, occurring precisely at the normal splicing sites.

It has been suggested that aberrant transcripts encode aberrant proteins with a dominant-negative effect (15). In our study the abnormal transcripts more frequently had deletions of exons 4-5-6 or of exons 4-5-6-7-8. Therefore, the major species of aberrant transcripts are not translated, since exon 5 contains the initiator codon for synthesis of the FHIT protein.

Analysis of genomic DNA from 28 informative patients for both loci showed LOH at the *FHIT* locus in 25% of the cases. Loss of heterozygosity more frequently involved the D3S1300 locus within intron 5 of the *FHIT* gene close to exon 5 and to the FRA3B region, whereas microsatellite instability (shift or appearance of extra alleles in tumour DNA compared with normal DNA) was present more frequently at the D3S1234 locus (Table II).

In conclusion, our molecular analysis showed that alterations involving the *FHIT* gene at the genomic level were present in 46.4% (13 of 28) of informative cases, and more than half of the analysed tumours had aberrant *FHIT* gene transcripts. The alterations in *FHIT* did not correlate with any of the clinicohistopathologic features analysed. Tumours showing aberrant transcripts or LOH at the *FHIT* locus were not located preferentially in any particular region of the colon and had no different histology when compared with tumours without *FHIT* gene alterations. We noted that aberrant transcripts of the *FHIT* gene and LOH were present in well-differentiated tumours, and the complete loss of the normal *FHIT* transcript occurred even in tumours with a low staging (Dukes stage A and B).

These observations suggest that *FHIT* gene inactivation may occur at an early phase of the carcinogenesis process; in fact, *FHIT* gene alterations have been described in breast hyperplastic lesions and in the premalignant condition of Barrett oesophagus (16, 17).

The role of the *FHIT* gene as a tumour suppressor gene is controversial; the high frequency of abnormalities found in our study and described by various authors could simply be related to the presence of a fragile locus inside the gene showing susceptibility to carcinogen-induced alterations.

The known function of pFHIT is diadenosine triphosphate activity (18), and it has been proposed that Ap3A and Ap4A (diadenosine tri-tetraphosphate) have various intracellular functions, including regulation of DNA replication and signalling stress responses. These molecules, in fact, stimulated DNA synthesis as measured with $^{3}H$ thymidine uptake and increased the expression of the early growth response gene EGR-1 (19). Apoptosis, in HL60 cells, is associated with a drop in Ap3A level (20). Baker & Jacobson (21) suggest that dinucleotides are involved in adaptive responses of mammalian cells to environmental stress such as hyperthermia, heavy metals (Cd), and ethanol and that deviation from the normal level may result in the inability of cells to adapt to external stresses. Loss of function of the *FHIT* gene could result in the constitutive accumulation of high levels of intracellular diadenosine polyphosphates and the stimulation of DNA synthesis and proliferation. However, some data do not support this hypothesis, since the transfection of *FHIT* DNA sequences into tumour cell lines does not change the in vitro cell proliferation rate but reduces the frequency of tumour formation in nude mice (22).

Recently, Burke et al. (23) have suggested the possible clinical implications of *FHIT* gene alterations in non-small cell lung carcinomas. They described a significant correlation between poor survival and LOH of at least one locus of the *FHIT* gene. In our series of colon adenocarcinoma cases we

did not find an association between the presence in the tumour of aberrant transcripts or *FHIT* genetic alterations and the relapse rate or the overall survival of the patient. The statistical power of our study is limited by the small sample size and, in particular, by the short follow-up period (36 months); a longer follow-up would further clarify the role of the *FHIT* gene in the molecular pathogenesis of colon carcinoma.

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