Absence of Msh2 Protein Expression Is Associated with Alteration in the FHIT Locus and Fhit Protein Expression in Colorectal Carcinoma

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Abstract

Frequent loss of Fhit expression has been reported in human gastrointestinal tract carcinomas; opinions remain divergent regarding Fhit expression in colorectal carcinoma (CRC) cases. Recent studies have suggested that Fhit inactivation can be a consequence of defects in mismatch repair proteins, particularly Msh2. Immunohistochemical analysis of Msh2 and Fhit protein expression in 62 CRC cases was performed. The same CRCs were examined for allelic loss at three loci within or near FHIT and for FHIT mRNA expression by reverse transcription-PCR amplification. Half of the 62 CRC cases were positive for Fhit protein. Fhit protein loss correlated significantly with the progression of carcinoma (P < 0.01) as well as lymph node metastasis (P < 0.05). Loss of Msh2 protein correlated significantly with loss of Fhit protein (P < 0.05) and FHIT locus alteration (P < 0.05). Loss of Fhit protein expression was observed in 50% of sporadic CRCs and was significantly more frequent in more advanced cancers. Interestingly, alteration of the fragile FHIT locus and loss of Fhit protein expression were significantly more frequent in sporadic CRCs lacking Msh2 protein, suggesting that this mismatch repair protein may be important in maintaining the integrity of the common fragile locus within the FHIT gene.

Introduction

Numerous recent studies have strengthened the position of the FHIT\(^1\) gene as a tumor suppressor gene including the demonstration of inhibition of tumorigenicity in nude mice by exogenous Fhit expression (1) and induction of apoptosis in cancer cells by overexpression of exogenous Fhit (2, 3). FHIT is involved in deletions on the short arm of chromosome 3 in various human gastrointestinal tract carcinomas. In CRC, Ohta et al. (4) reported initially that aberrant transcripts of the FHIT gene in three of eight (38%) CRC cases. In addition, several reports have described the important role that the FHIT gene plays in either oncogenesis or the progression of CRC (5–7). In contrast, Thiagalingam et al. observed very infrequent LOH at the FHIT locus in 29 normal FHIT RT-PCR products in 94% of CRCs and concluded that there was no evidence that the FHIT gene was involved in CRC carcinogenesis (8). Thus, the clinicopathological significance of FHIT alterations in CRC has yet to be clearly elucidated. The purpose of this study was to assess the frequency of FHIT alterations in sporadic CRC and to determine whether there was an association between Msh2 absence and FHIT alterations. Fong et al. (9) recently investigated the role of the FHIT gene in carcinogen induction of neoplasia by producing F1 mice with a FHIT allele inactivated (+/−). They reported that 100% of the FHIT (+/−) developed adenoma or papilloma of the forestomach, and >50% exhibited tumors of sebaceous gland, demonstrated that carcinogen induces a phenotype very much similar to the human Muir-Torre familial cancer syndrome (9). The majority of Muir-Torre familial cancer syndrome cases exhibit MSI and usually germ-line mutations in the MSH2 gene (10). Additionally, Hilgers et al. (11) reported that the occurrence of HDs at FHIT exon 5 flanking microsatellite loci occurs strikingly frequently in pancreatic cancers exhibiting the “replication error” phenotype, indicative of defective mismatch repair. These reports suggested that the FHIT gene could be a target of damage in mismatch repair deficient tumors, especially in those with Msh2 deficiency, leading to loss of Fhit protein. Because the incidence of Msh2 alterations is relatively high in sporadic CRC, we investigated the relationship between Msh2 and Fhit expression as an approach for understanding the mechanism of inactivation of FHIT in human cancers.

Materials and Methods

Cancer Cases and Tissues. Sixty-two Japanese CRCs were examined. Cancer patients underwent surgery at the Medical Institute of Bioregulation Kyushu University, Beppu and Oita Prefectural Hospital, Oita, Japan. There were 37 male and 25 female patients with an average age of 66 and a range from 26 to 88 years. There was no familial history in 62 CRC cases. Among the 62 cases, 17, 19, 17, and 9 were classified as Dukes’ stage A, B, C, and D, respectively. The clinicopathological classifications are shown in Table IA.

DNA Extraction and Analysis of MSI. At the time of surgery, carcinoma tissues and the corresponding normal tissues were snap frozen and stored at −80°C until use. Genomic DNA was extracted by the ultra-centrifuge method. Sequence-tagged site markers used were D3S1312 centromeric to FHIT, D3S1295 telomeric to FHIT, and D3S1300 within FHIT. The sequences of primers were obtained from the Genome Database, and reverse primers were labeled by either 5′-fluorescein phosphoramidite or 5-tetrachlorofluorescein phosphoramidite as described in our previous study (12).

Immunohistochemical Detection of Msh2 and Fhit Protein. Msh2 protein in 44 paraffin-embedded CRC and corresponding normal tissues were examined by immunohistochemical analysis as described previously with monoclonal serum specific for human Msh2 protein (DAKO, Tokyo, Japan; Refs. 13, 14). We scored the expression as negative when <10% of the carcinoma cells were stained in an examined area of a specimen. The staining for Msh2 and Fhit was performed on adjacent sections. Fhit immunohistochemical staining was performed as described previously (12) with a polyclonal serum specific for human Fhit (Zymed Laboratories, Inc., South San Francisco, CA).

Clinical and Pathological Comparison of Fhit Positive and Negative Cases. Fhit positive and negative cases were compared regarding histological differentiation, depth of tumor invasion, lymphatic permeation, vascular vessel invasion, lymph node metastasis, and Dukes’ stage of disease.

RT-PCR Analysis of FHIT mRNA Expression. The FHIT gene is encoded by 10 exons in a 1.1-kb transcript. FHIT mRNA expression was analyzed in 62 cases by PCR amplification using nested primers as described previously (15).
MSH2 AND FHIT EXPRESSION IN COLORECTAL CARCINOMA

Table 1A Correlation of Fhit protein expression with clinical and pathologic features of CRCs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Positive (n = 31)</th>
<th>Negative (n = 31)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>14</td>
<td>0.04</td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Dukes’ stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>13</td>
<td>4</td>
<td>0.01</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Table 1B Multivariate analysis on the determination of the patient prognosis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Regression coefficient</th>
<th>SE</th>
<th>Relative risk (95% confidence interval)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes</td>
<td>-1.3363</td>
<td>0.6954</td>
<td>8.5400 (2.6183-27.8577)</td>
<td>0.009</td>
</tr>
<tr>
<td>Fhit</td>
<td>-1.3363</td>
<td>0.6954</td>
<td>8.5400 (2.6183-27.8577)</td>
<td>0.0387</td>
</tr>
</tbody>
</table>

* A significant difference was observed between the Dukes’ A+B groups and the Dukes’ C + D groups. Fhit expression is not significantly correlated with histology, depth of tumor invasion, lymph vessel permeation, or vascular vessel permeation.

Statistical Analysis. The relationship between the clinicopathological variables and FHIT locus on protein alterations was tested by Fisher’s exact test; P < 0.05 was considered to indicate statistical significance. The relationship between prognosis and the expression of Fhit in CRC is analyzed by the Cox-Mantel test and Kaplan-Meier analysis. Multivariate analysis on the determination for the patient prognosis was also performed.

Results

Clinicopathological Significance of Fhit Expression in Sporadic CRC. A summary of the results of immunohistochemical analysis of Fhit expression and a photograph of a representative case are shown in Table 1A and Figs. 1 and 2, respectively. There were 31 Fhit-positive and 31 negative CRCs. Regarding clinicopathological variables, 17 of 25 (68%) cases with lymph node metastasis were negative for Fhit expression, whereas 23 of 37 (62%) cases without lymph node metastasis were Fhit positive (P = 0.04). In addition, a significant 18 of 26 (69%) of Dukes’ C and D cases were Fhit negative, whereas 23 of 36 (64%) Dukes’ A and B cases were Fhit positive (P = 0.01). Moreover, prognosis of Fhit-negative cases was much poorer than Fhit-positive cases with statistical significance in Fig. 3 (P = 0.03). The multivariate analysis is shown in Table 1B. Fhit expression was the second and independent determinant factor for patient prognosis (P = 0.04). There was no significant correlation of other clinicopathological factors with Fhit expression.

Alteration of the FHIT Locus. LOH and HD were assessed at microsatellite markers within and flanking the FHIT gene. Among 62 cases, 43 (69%) exhibited either LOH and/or HD. We observed LOH in 4 (6%), 10 (16%), and 9 (15%) of the cases at D3S1312, D3S1300, and D3S1295, respectively. HD was observed in 8 (13%), 17 (27%), and 11 (18%) of the cases at D3S1312, D3S1300, and D3S1295, respectively.

RT-PCR analysis showed that 13 cases (42%) and 5 cases (16%) exhibited no expression or aberrant product in the 31 Fhit-negative and 31 Fhit-positive cases, respectively (Table 2 and Fig. 4), and the difference was statistically different (P < 0.05). The incidence of genomic alteration was 26 (84%) and 17 (55%) in 31 Fhit-negative and 31 Fhit-positive cases, respectively (P < 0.02).

Expression of Msh2 and Correlation with FHIT Alterations. Of CRC tissue specimens, 85% (53/62) were positive for expression of Msh2 protein (Fig. 2), whereas the remaining 15% (9/62) showed no detectable expression. All of the corresponding normal colon tissue specimens were positive for Msh2 expression. Regarding correlation between Msh2 and Fhit protein expression (Fig. 3), 30 (97%) of 31 Fhit protein-positive cases showed positive for Msh2 expression, whereas 8 of 9 Msh2-negative cancers were Fhit negative. The difference was statistically significant (Table 3; P = 0.01).

Interestingly, in 9 cases with no expression of Msh2, all 9 (100%) of the cases showed genomic alterations at the FHIT locus (P = 0.04). At the D3S1300 locus, 6 of 9 Msh2-negative cases showed HD. At the D3S1312 locus, 2 cases exhibited LOH, whereas 2 other cases showed HD. At D3S1313, there were 2 cases with LOH and 3 cases with HD.

MSI was observed in 6 of 62 cases. One of 9 Msh2-negative CRCs showed MSI at D3S1295, whereas 5 Msh2-positive cases showed MSI. These 5 MSI cases must have occurred because of mismatch repair deficiencies in tumors other than Msh2.

Aberrant FHTI transcripts were observed in 24 of 62 (39%) cases. Among 24 cases with aberrant transcripts, 10 (32%) were positive for Fhit proteins, whereas 14 (45%) were Fhit negative. Among 9 Msh2 negative cases, 3 (30%) cases showed an aberrant FHTI transcript.

Discussion

Significance of Fhit Loss in CRC. In the clinicopathological analysis of the expression in the 62 cases, the loss of Fhit protein was associated with advanced stage of the disease and with frequent occurrence of lymph node metastasis. This correlation suggests a role for the Fhit in the progression of CRC as follows: the loss of Fhit

![Fig. 1. Top, Msh2 expression in CRC. Immunohistochemical staining with anti-Msh2 antibody in CRC cells (left) and normal mucosa (right). Positive staining is observed in the nuclei of cells in normal tissue. Bottom, magnification of boxed area in top figure. A magnified area showing the difference in expression of Fhit protein in the normal versus cancer portions of the section.](image-url)
protein is intimately associated with the development of CRC but not with its initiation. Our conclusion and interpretation are supported by the findings of Hao et al. (7) who reported reduced expression of Fhit in a small proportion of colorectal precancerous lesions and in a larger fraction of primary and metastatic colorectal cancers. Therefore, it was suggested that Fhit plays a role in development and progression in sporadic CRC. Moreover, Croce et al. (16) concluded that alterations in the FHIT gene occur quite early in the development of cancers associated with environmental carcinogens such as tobacco and alcohol. In fact, we reported previously frequent alteration of FHIT in precancerous lesions of the esophagus especially in cases strongly exposed to those carcinogens (12). Other cancers exhibited Fhit inactivation as a later event, possibly associated with progression to more aggressive neoplasia. Our current study indicates that loss of Fhit protein appears to be related to a progression of CRC.

**Correlation between the Loss of Msh2 Protein and Alteration of FHIT.** Chaves et al. (13) reported frequent loss of Msh2 protein is observed in human sporadic CRC. In the present study, 9 of 62 (15%) CRC cases exhibited altered expression of Msh2, which significantly correlated with loss of Fhit protein. Moreover, a significant correlation was observed between the frequency of LOH and/or HD in FHIT and altered Msh2 protein expression. These results suggested that loss of the Msh2 repair function leads to enhanced damage of the fragile region within FHIT.

**Table 2** Correlation of Fhit protein expression with FHIT mRNA expression and genomic alterations

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Positive (n = 31)</th>
<th>Negative (n = 31)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>26</td>
<td>18</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Absent or aberrant</td>
<td>5</td>
<td>13</td>
<td>P = 0.016</td>
</tr>
<tr>
<td>Genomic alteration*</td>
<td>14</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

* LOH and/or HD in at least one of three markers.

**Table 3** Correlation of Msh2 protein expression with alterations of the FHIT locus and protein

<table>
<thead>
<tr>
<th>FHIT alterations*</th>
<th>Msh2 protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>(n = 53)</td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fhit protein</th>
<th>Positive</th>
<th>Negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>30</td>
<td>1</td>
<td>P = 0.01</td>
</tr>
<tr>
<td>Negative</td>
<td>33</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

* LOH and HD. Significant correlations were observed between Fhit and Msh2 expression and also between the FHIT locus alterations and Msh2 expression.
Interestingly, the repetitive elements are frequent in introns 4 and 5 of the FHIT gene, which we sequenced previously (17, 18). (CA)n and (A)n repeats can cause of replication errors. In Msh2-negative CRC cases, such errors cannot be repaired during replication. Such repetitive sequences are not located in Fhit coding exons, and it is not clear that such errors could affect FHIT alterations. It is also not clear how loss of a mismatch repair protein could lead to the large hemizygous and HDs.

In conclusion, the incidence of Fhit protein loss in CRC was significantly associated with advanced disease at Dukes’ C and D stages (P < 0.01) and with lymph node metastasis (P < 0.05). Loss of expression of a mismatch repair protein, Msh2, was significantly correlated with loss of Fhit expression as well as with genomic alterations of the FHIT region.

References


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