Reduced Fhit expression is associated with mismatch repair deficiency in human advanced colorectal carcinoma

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A candidate tumour suppressor gene, Fragile Histidine Triad (FHT), was identified at chromosome 3p14.2 spanning the FRA3B common fragile site (Ohta et al, 1996). The FHT gene consists of 10 exons, which distribute over a genomic region of >1 Mb. The Fragile histidine triad (Fhit) protein has been characterised as an Ap3A hydrolase molecule, which cleaves the Ap4A substrate that may be involved in the control of DNA replication and the cell cycle (Ohta et al, 1996). Frequent abnormal transcripts were found in a variety of human cancers including those of the digestive tract, lung, breast, and head and neck (Ohta et al, 1996; Croce et al, 1999; Kitamura et al, 2001). The majority of these abnormalities include aberrant mRNA transcripts, with the absence of one or more exons within the mRNA. Genomic analysis demonstrated frequent allelic loss and homzygous deletions (Ohta et al, 1996; Croce et al, 1999).

In CRCs, Ohta et al (1996) reported that aberrant transcripts of the FHIT gene were detected in three of eight tumours by nested RT–PCR. In contrast, Thiagalingam et al (1996) reported that aberrant transcripts were rare using RT–PCR, suggesting that FHIT is inactivated by an unusual mechanism or that it plays a role in relatively few colorectal tumours. Other reports suggested that Fhit does indeed play a role in the development and progression of colorectal carcinomas (CRCs) (Hibi et al, 1997; Hao et al, 2000; Luceri et al, 2000; Mori et al, 2001).

Defects in the DNA mismatch repair (MMR) system are involved in the carcinogenesis and tumour progression of sporadic and inherited human cancers (Eshleman et al, 1996; Kinzler and Vogelstein, 1996). MMR deficiency leads to the accumulation of base-base mismatches and short insertion/deletion mispairs, generated as a consequence of DNA replication errors and homologous recombinations. Inherited mutations of the MLH1 and MSH2 genes have been demonstrated as the cause of more than 90% of hereditary non-polyposis colorectal cancers (HNPCC) (Peltomaki and Vasen, 1997). MMR genes are also involved in the development of a subset of sporadic colorectal, gastric and endometrial tumours (Duggan et al, 1994; Halling et al, 1999; Ward et al, 2001). MMR deficiency, identified by the presence of microsatellite instability (MSI), occurs in approximately 10–15% of sporadic CRCs (Kinzler and Vogelstein, 1996). Most sporadic CRCs with MSI have been demonstrated to be caused by somatic hypermethylation of the MLH1 promoter region, resulting in the down-regulation of MLH1 gene expression (Herman et al, 1998). Recent data have revealed that immunohistochemistry is an accurate screening technique to identify MMR deficient tumours (Thibodeau et al, 1996; Marcus et al, 1999).

Recently, Fong et al (2000) have demonstrated that NMBA (N-nitrosomethylbenzylamine) exposure caused a spectrum of visceral
and skin tumours similar to Muir-Torre syndrome, caused by a
deficiency in a MMR gene, in Fhit-deficient mice, and suggested that
the FHIT gene may be a target of damage in a fraction of
mismatch deficient tumours. Moreover, Mori et al (2001) reported that
the loss of Msh2 protein is significantly correlated with the
loss of Fhit expression in human CRCs. However, the relationship
between Fhit and the MMR gene (both Msh2 and Mlh1) expres-
sion has not been previously studied in detail using clinical
samples.

In this study, we examined the immunohistochemical expression
of Fhit, Msh2, Mlh1, and p53 in advanced CRCs to explore the
hypothesis that the Fhit inactivation is a frequent result of MMR
deficiency.

MATERIALS AND METHODS

Patient samples

Tumour blocks were obtained from 52 patients, who underwent
colorectomy at Tottori University Hospital between 1992 and
1996. Patients with familial adenomatous polyposis (FAP) were
excluded, but family history was not an exclusion criterion; there-
fore, some cases of HNPCC may have been included in the study
population. The ages of the 27 male and 25 female patients ranged
from 51 to 90 years (mean ± SD: 67.4 ± 13.2 years). Pathologically,
all of the tumours were advanced adenocarcinomas (19 well-differ-
etiated, 17 moderately-differentiated, and 16 poorly-
differentiated). Nineteen cases had right sided tumours and 33
cases had left sided tumours. Right-sided lesions were defined as
those confined to the coecum or ascending or transverse colon,
whereas left-sided lesions were defined as being confined to the
descending colon, sigmoid colon, or rectum. On the basis of
Dukes’ classification, four were in stage A, 12 in stage B, 21 in stage
C, and 15 in stage D. Lymph node metastases were present in 34 of
the 52 (59.6%) patients. Histological classification was made
according to the criteria of the Colorectal Cancer Study Group
of Japan (Colorectal Cancer Study Group of Japan, 1998). All diag-
noses of pathological specimens were verified by two experienced
pathologists (H.A. and H.I.). All the cases were analysed anon-
ymously, i.e., all the specimens were given new numbers without
names. Institutional Review Board approval was obtained.

Immunohistochemical staining

Paraffin-embedded, 4 μm-thick sections were immunohistochemi-
cally stained with anti-FHIT rabbit polyclonal antibody (IBL,
Gunma, Japan; dilution 1:100), anti-MSH2 mouse monoclonal
antibody (FE11, Oncogene Research Products, Cambridge, MA,
USA); dilution 1:100), anti-MLH1 mouse monoclonal antibody
(G168-15, PharMingen, San Diego, CA, USA; dilution 1:50), and
anti-p53 mouse monoclonal antibody (DO-7, Dakopatts, Copen-
hausen, Denmark; dilution 1:50) using the avidin-biotin-peroxidase
complex technique. Immunohistochemical staining was performed
as described below. In brief, after deparaffinising in xylene and
dehydrating in ethanol, the sections were immersed in a citrate
buffer (0.01 M, pH 6.0) and heated in a microwave oven for
15 min to retrieve antigens, then incubated with the primary anti-
body overnight at 4°C. As a negative control, the primary antibody
was replaced with normal serum IgG in a similar dilution. The
detection reaction followed the Vectastain Elite ABC kit protocol
(Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine
was used as a chromogen, and methylgreen or haematoxylin was
used as a counterstain. The sections were then incubated with
biotinylated anti-rabbit IgG and avidin-biotin-peroxidase and
visualised using diaminobenzidine tetrahydrochloride. The immu-
nohistochemical expressions were evaluated by two independent
observers (H.A. and K.Y.). Immunohistochemical analysis was
performed in a blinded manner with respect to the clinical infor-
mation.

Assessment of Fhit immunostaining

The Fhit expression was graded for both the extent and the intensity
of immunopositivity as described previously Hao et al. (2000). The
extent of positivity was scored as follows: 0, <5%; 1, >5 – 25%; 3,
>50 – 75%; and 4, >75% of the colonic epithelial cells in the respec-
tive 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. Scores 9 – 12 were defined
as preserved or strong staining pattern, scores 0 – 4 were defined as
markedly reduced or lost expression, and scores 5 – 8 were defined
as intermediate staining pattern.

Assessment of Msh2 and Mlh1 immunostaining

Normal tissue adjacent to the tumour was used as an internal posi-
tive control. The normal staining pattern for both hMlh1 and
hMsh2 was nuclear. Tumour cells that exhibited an absence of
nuclear staining in the presence of non-neoplastic cells with nuclear
staining were considered to have an abnormal pattern.

Assessment of p53 immunostaining

Five representative fields were examined, and a total of 1000
tumour cells (200 for each field) were counted under the micro-
scope with a high power (× 200) objective. A distinct nuclear
immunoreaction was assessed as positive. In this study, the speci-
mens were regarded as p53-positive when over 25% of the
tumour cells showed positive signals.

Statistical evaluation

Statistical analysis was performed by the Fisher’s exact test. P < 0.05
was considered significant.

RESULTS

Fhit expression in the normal mucosa and advanced
cancers

By immunohistochemical staining, all the normal colonic epithelia
showed strong cytoplasmatic expression of the Fhit protein from
the basal cells to the luminal differentiated cells (Figure 1A); these
served as internal controls. Smooth muscle cells and inflammatory
mononuclear cells were positive at various intensities and to
various degrees.

Reduced or absent Fhit expression was noted in 18 of the 52
(34.6%) advanced CRC cases, preserved in 25 (48.1%) CRCs and
intermediate in nine (17.3%) CRCs (Table 1, Figure 1A,B). The
intensity of the staining of the colorectal carcinoma cells was deter-
mimed by comparing it to that of the normal colonic epithelial cells
found within the same specimen (Figure 1A).

We analysed the relationship between these results and the clin-
ico pathological data (tumour location, histological differentiation,
Dukes’ stage, lymph node metastasis, and p53 expression). Decreased staining or lack of staining for Fhit was detected in 2
of 19 (10.5%) well-differentiated cancers, in three of 17 (16.7%)
moderately-differentiated cancers, and in the 13 of 16 (81.3%)
poorly-differentiated cancers. The incidence of reduced Fhit expres-
sion in CRCs was significantly highest in the poorly-differentiated
histology (P<0.0001; Table 1). However, no significant associa-
tions were found among the Fhit expression and other clinico pathological parameters.
Correlation of Fhit expression with MMR gene expression

The expression of both Mlh1 and Msh2 proteins was without exception nuclear. In the normal mucosa, they were detected predominantly in the areas of active proliferation, such as the germinal centres of the lymphoid follicles and the lower portions of the normal colonic crypts (Figure 2A,C). Normal stromal cells such as fibroblasts and endothelial cells also showed nuclear positivity for both the proteins. We demonstrated that 19 of the 52 (36.5%) tumours had reduced expression levels of the Mlh1 protein (Figure 2B), whereas five (9.6%) carcinomas had reduced expression levels of the Msh2 protein (Figure 2D). Reduced expression levels of both proteins were observed in three of the 52 (5.8%) specimens (Table 2). In addition, loss of nuclear Mlh1 or Msh2 expression was more frequently associated with poor differentiation ($P=0.0128$) and right-sided location ($P=0.0184$) (Table 1). However, there was no significant difference in other clinicopathological parameters (age, gender, Dukes stage, node metastasis, and p53 expression). Among the tumours with reduced or absent Fhit expression, 72.2% (14 of 18) had loss of nuclear Mlh1 or Msh2 expression compared with only 20.6% (seven of 34) of the preserved or intermediate Fhit expression tumours ($P<0.0001$) (Table 2).

DISCUSSION

Alterations and abnormal transcripts of the FHIT gene have been reported in a number of primary human tumours, including CRCs (Ohta et al, 1996; Croce et al, 1999; Kitamura et al, 2001). However, some of the data are conflicting in CRCs (Hao et al, 2000; Hibi et al, 1997; Luceri et al, 2000; Mori et al, 2001; Thiagalingam et al, 1996). Recently, it was reported that alterations in the FHIT locus detected by DNA and/or reverse transcription-PCR analysis correlated with a loss of Fhit protein expression in lung, cervical, and oesophageal carcinomas (Birrer et al, 1999; Mori et al, 2000; Sozzi et al, 1998). These results indicated that FHIT gene alteration can be simply detected by immunohistochemical analysis of tumour specimens. In this study, we observed frequent abnormal Fhit protein expression in poorly-differentiated CRCs, with 77% of the specimens demonstrating a decrease in, or lack of, Fhit

Table 1  Relationship between Fhit and MMR protein expression and clinicopathological findings in advanced colorectal carcinoma

| Histological type | Fhit protein expression | Mlh1/Msh2 expression | $P$ |
|-------------------|-------------------------|----------------------|-----|
|                  | preserved/intermediate  | reduced or absent    |     |
| well              | 19                      | 17                   | 2   |
| moderately        | 17                      | 14                   | 3   | $<0.0001$ |
| poorly            | 16                      | 3                    | 13  |
| Tumour localization |                        |                      |     |
| right side       | 19                      | 11                   | 8   | NS |
| left side        | 33                      | 23                   | 10  |
| Dukes’ stage     |                         |                      |     |
| A                 | 4                       | 4                    | 0   | NS |
| B                 | 12                      | 8                    | 4   | 6   |
| C                 | 21                      | 10                   | 11  | 10  |
| D                 | 15                      | 12                   | 3   | 12  |
| Lymph node metastasis |                  |                      |     |
| +                 | 34                      | 20                   | 14  | NS |
| −                 | 18                      | 14                   | 4   | 11  |
| p53 expression   |                         |                      |     |
| +                 | 35                      | 24                   | 11  | NS |
| −                 | 17                      | 10                   | 7   | 9   |

Right-sided lesions were defined as those proximal to the splenic flexure. NS: not significant.

Correlation of Fhit expression with MMR gene expression

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DISCUSSION

Alterations and abnormal transcripts of the FHIT gene have been reported in a number of primary human tumours, including CRCs (Ohta et al, 1996; Croce et al, 1999; Kitamura et al, 2001). However, some of the data are conflicting in CRCs (Hao et al, 2000; Hibi et al, 1997; Luceri et al, 2000; Mori et al, 2001; Thiagalingam et al, 1996). Recently, it was reported that alterations in the FHIT locus detected by DNA and/or reverse transcription-PCR analysis correlated with a loss of Fhit protein expression in lung, cervical, and oesophageal carcinomas (Birrer et al, 1999; Mori et al, 2000; Sozzi et al, 1998). These results indicated that FHIT gene alteration can be simply detected by immunohistochemical analysis of tumour specimens. In this study, we observed frequent abnormal Fhit protein expression in poorly-differentiated CRCs, with 77% of the specimens demonstrating a decrease in, or lack of, Fhit
protein staining. This frequency of abnormal Fhit expression was similar to that observed in poorly-differentiated CRCs by Hao et al. (2000). Therefore, these results suggest that abnormal Fhit expression is associated with decreasing degrees of differentiation in CRCs. However, we found no correlation between the Fhit expression and any of the clinico-pathological parameters including p53 expression.

Inactivation of MMR genes is a recently described alternate pathway in cancer development and progression (Eshleman and Makowitz, 1996; Kinzler and Vogelstein, 1996). MMR deficiency is present in 10–15% of sporadic CRCs (Kinzler and Vogelstein, 1996), and is the underlying cause of more than 90% of HNPCC (Peltomaki and Vasen, 1997). Previous studies demonstrated that immunohistochemical analysis of the expression of Mlh1 and Msh2 could be an accurate and rapid screening procedure for the identification of MMR gene alterations (Thibodeau et al., 1996; Marcus et al., 1999). We found abnormal Mlh1 or Msh2 expression in 21 of the 52 (46%) CRCs using immunohistochemical methods. This frequency of abnormal MMR protein expression was more frequent than that observed in CRC by other investigators (Kinzler and Vogelstein, 1996). In the assessment of MMR protein expression, we used almost the same criteria used by other groups (Marcus et al., 1999; Ward et al., 2001). In addition, it was reported that sporadic CRCs with MSI were more frequent in the poorly differentiated phenotype (Ward et al., 2001). In our study, the ratio of poorly differentiated CRCs was high compared with previous reports (Kinzler and Vogelstein, 1996; Marcus et al., 1999; Ward et al., 2001). Therefore, high frequency of abnormal MMR protein expression might be mainly due to the different distributions of histological differentiation. Moreover, we found a significant correlation between the MMR protein expression and histological differentiation and tumour location. These results were also consistent with previous reports (Kinzler and Vogelstein, 1996). In the present study, MMR deficiency was mainly due to the loss of Mlh1 expression, suggesting that MLH1 hypermethylation is the predominant mechanism (Herman et al., 1998).

### Table 2: Relationship between Fhit and MMR protein expression in colorectal carcinoma

| Fhit expression | n  | Preserved/intermediate | Reduced or absent |
|-----------------|----|------------------------|-------------------|
| Mlh1 (+), Msh2 (+) | 31 | 27                     | 4                 |
| Mlh1 (−), Msh2 (+) | 16 | 4                      | 12                |
| Mlh1 (+), Msh2 (−) | 2  | 1                      | 1                 |
| Mlh1 (−), Msh2 (−) | 3  | 2                      | 1                 |

Reduced Fhit expression is associated with loss of MMR expression: \( P < 0.0001 \).
Recently, it has been reported that by NMBA (N-nitrosomethylbenzylamine) exposure, Fhit-deficient mice developed a spectrum of visceral and skin tumours similar to Muir-Torre syndrome, caused by a deficiency in a MMR gene (Fong et al., 2000). A large subgroup of MTS cases exhibits MSI and germline mutations in the MLH1 or MSH2 gene (Krusce et al., 1998). In addition, it was previously observed that human pancreatic cancers and cell lines with high MSI frequently had homozygous addition, it was previously observed that human pancreatic carcinomas and support this hypothesis. Mori et al. (2001) reported an association between Msh2 absence and FHit alterations in CRCs but did not analyse Mlh1 expression. Moreover, as the mechanisms of this hypothesis, they proposed that the repetitive elements, such as (CA)n and (A)n repeats, in introns 4 and 5 of the FHit gene could be a target of damage in MMR deficient tumours.

Thus, we noted an association between Fhit and MMR protein expression, but it could be due to the result of an association between Fhit and MMR protein expression and poorly differentiation.

In conclusion, we have demonstrated that reduced Fhit expression was associated with the loss of MMR protein expression in advanced CRCs. In combination with previous reports, this result supports the possibility that FHit gene alterations could be caused by a deficiency in a MMR gene. Studies that look into the regulation of Fhit and MMR protein expression in cancer may offer a new insight into colorectal carcinogenesis and plausibly, chemopreventive pathways.

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