FHIT Is Up-Regulated by Inflammatory Stimuli and Inhibits Prostaglandin E2–Mediated Cancer Progression

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Abstract

The FHIT gene is known to be susceptible to environmental carcinogens. Formation of prostaglandin E2 (PGE2) is catalyzed by cyclooxygenase-2 (COX-2) and may influence malignant phenotype in colorectal cancer. We explored whether FHIT might play a role in progression of colorectal cancer through inflammation-associated PGE2 activity. Immunohistochemical study of COX-2 and FHIT expression was done in 92 colorectal cancer tumors. We also used a FHIT-expressing cancer cell line (H460) induced by ponasterone A and two FHIT small interfering RNA–treated colorectal cancer cell lines (CCK81 and DLD1). After PGE2 stimulation, we compared synthesis of PGE2 (ELISA assay) and cell proliferation [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]. Immunohistochemistry showed a significant association between COX-2 and FHIT expression in colorectal cancers (P < 0.01). In a subset of 41 COX-2–expressing tumors, 12 FHIT- tumors showed deeper cancer invasion than 29 FHIT+ tumors (P < 0.01). Experimental study, however, showed there was no direct interaction between FHIT and COX-2. Considered with results from another experiment with epidermal growth factor receptor (EGFR), we hypothesize that FHIT and COX-2 might be regulated by a common molecule, such as EGFR. Additionally, there was an inverse and direct correlation between PGE2 synthesis and FHIT in vitro, suggesting that FHIT’s postulated antiaggressive effect on tumor goes through PGE2 but not COX-2. Loss of FHIT expression in colorectal cancer suggests higher malignant potential. We conclude that FHIT suppressed cancer cell proliferation in this malignancy by directly inhibiting synthesis of PGE2 but not affecting that of COX-2. (Cancer Res 2006; 66(5): 2683-90)

Introduction

The FHIT gene encompasses the environmental carcinogen–sensitive common fragile site, FRA13B. The gene is frequently hemizygozously or homozgyously deleted in several kinds of cancers (1–5), including environmental carcinogen–related cancers, such as those of the lung (6–10) and esophagus (11). Numerous studies have reported altered FHIT expression not only in advanced tumors but also in precancerous lesions, suggesting that FHIT mutation or deletion can be an early event in carcinogenesis (12). Abundant expression of FHIT protein has been shown to cause apoptosis in various cancer-derived cell types (13, 14), suggesting that wild-type FHIT may act as a tumor suppressor against cancer cells. We, therefore, hypothesized that FHIT might be a susceptible gene whose reconstitution could act against environmental carcinogens.

For a number of cancers associated with external or internal carcinogens, including colorectal cancer, inflammation is considered to be a key event affecting tissue. Prostaglandin E2 (PGE2) is considered to play a key role in inflammation through synthesis from arachidonic acid by cyclooxygenase-2 (COX-2). In the current study, we first investigated expression of FHIT and COX-2 with immunohistochemical techniques in 92 colorectal cancer tumors. Although we found a positive correlation between them (P < 0.001), there was no direct relationship in in vitro experiments. Then, we focused on a possible relationship between FHIT and PGE2.

We clarified whether induction of PGE2 in cancer cells promotes cellular proliferation and whether FHIT protein regulates PGE2 induction. PGE2 production and cellular proliferation were compared for cells that overexpressed FHIT and control cells that did not express FHIT, respectively. In addition, as a confirmation experiment, we compared FHIT-knockdown down cells and control cells. Consequently, we discovered that FHIT inhibits PGE2 activation directly.

Concerning the susceptible marker for colorectal cancer, we predict that loss of FHIT expression and positive expression of PGE2/COX-2 indicate worse malignant behavior in colorectal cancer tumors. In addition, our findings suggest that delivery of FHIT protein may be a novel molecular therapy that could possibly prevent inflammation-related malignant progression.

Materials and Methods

Colorectal cancer tissue samples. A total of 92 colorectal adenocarcinomas excised at our institution and the Department of Surgery, Oita Prefectural Hospital, Oita, Japan were used after receiving approval from each institutional ethics committee and confirming that informed consent had been obtained from all subjects. Samples represented 53 male and 39 female patients with a mean age of 66 years (range, 15–88 years). None of the patients had a familial history of colorectal cancer.

Immunohistochemistry. Paraffin-embedded cancer tissue specimens and corresponding normal tissue specimens were examined by immunohistochemical analysis with COX-2 monoclonal antibody (C22420, Transduction Laboratories, San Diego, CA) and FHIT polyclonal antiserum (Zymed Laboratories, Inc., Koto-Ku, Tokyo, Japan), as previously described (11). All sections were examined independently by three investigators (K.M., H.I., and M.M.). We scored tumors as expression negative for COX-2 or FHIT when <10% of carcinoma cells were stained in an examined area of a specimen. Staining for COX-2 and FHIT was done on adjacent sections. All cases were examined for histologic differentiation, depth of tumor invasion,
lymphatic permeation, vascular vessel invasion, lymph node metastasis, and Duke's disease stage.

**Cell culture and inducible FHIT transfectants.** We transfected H460 cells with the pVgRXR vector and H460 cells with the pND vector (control) provided by Dr. Jennifer Pietenpol (Vanderbilt University). The cells were cultured in DMEM medium with 10% fetal bovine serum (FBS), G418, and Zeocin. After administration of 10 μmol/L ponasterone A, the ability to induce the FHIT transgene was confirmed by Western blot analysis. Using this clone, we compared COX-2 expression in FHIT⁺ cells and FHIT⁻ cells after lipopolysaccharide (LPS) stimulation.

**Measurement of PGE₂ production by ELISA.** Induction of PGE₂ synthesis by 0.1 and 1.0 μg/mL LPS, 10 ng/mL interleukin-1β (IL-1β), and 10 ng/mL phorbol 12-myristate 13-acetate (PMA) was done as previously described (15) for FHIT transfectant and control cells.

At 24 hours after ponasterone A administration, culture medium was replaced with fresh 10% FBS medium containing 1.0 μg/mL LPS, 10 ng/mL IL-1β, and 10 ng/mL PMA. We did experiments four times for PGE₂-inducing stimulation and obtained each average score with SE using Student’s t test analysis. Cells were then incubated for an additional 48 hours before PGE₂ expression was determined using the enzyme immunoassay kit-monoclonal antibody (Cayman Chemical Co., Ann Arbor, MI), according to the manufacturer’s instructions.

**Comparison of cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** We compared proliferation rate using an [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay after ponasterone A administration of FHIT-expressing cells as well as control cells that did not express FHIT. Induced FHIT expression was initially elevated 24 hours after ponasterone A administration, and we added ponasterone A every 24 hours until 72 hours later. At 24 hours before the experiment, we initially administered ponasterone A, and then we started inducing PGE₂ expression in both FHIT-expressing cells and control cells by stimulation with 1.0 μg LPS at 0 hour. We did all experiments four times for each stimulant; therefore, we calculated 12 data points at each time and for each cell type to calculate the average and SE. In brief, 20-μL suspensions of 2×10⁵ cells treated with 1.0 μg/mL LPS, which showed a significant difference of PGE₂ production between FHIT⁺ and FHIT⁻, were aliquoted into microtiter plate wells. The untreated cells were used as controls for nonspecific dye reduction. After incubation for 0, 4, 24, 48, or 96 hours in a humidified 5% CO₂ atmosphere, plates were spun at 700 × g, the supernatants were discarded, and 20-μL MTT solution (10 μmol/L) was added to each well except for those containing nontreated controls. After an additional hour of incubation, 150 μL DMSO was added to every well to extract the formazan form of MTT. Formazan absorbance was measured at 565 nm; values are represented as absorbance per mg protein. Under LPS stimulation, increased expression of COX-2 was observed independently from FHIT expression (Western blotting, described below).

**Selection of small interfering RNA-transfected colorectal cancer cells by quantitative real-time reverse transcription-PCR.** The FHIT gene is encoded by 10 exons in a 1.1-kb transcript. We examined and quantified FHIT mRNA expression in the colorectal cancer cell lines LoVo, HT29, DLD-1, COLO320DM, COLO205, COLO201, and CCK-81 by quantitative reverse transcription-PCR (RT-PCR) assay (LightCycler 2000, Roche Diagnostic, Tokyo, Japan) using nested primers as described previously (16).

**Small interfering RNA transfection.** We used DLD-1 and CCK-81 cell lines, both of which express FHIT protein, as representative colorectal cancer cell lines. The expression vector pSilencerTM3.1-H1 hygro (Ambion, Inc., Austin, TX) was used for expression of small interfering RNA (siRNA). A hairpin siRNA designed to target the FHIT gene (5’-GGAAAGCGUGAGACUUCACTT-3’, sense; 5’-UGAAAGGUCGUCCGC-UUCCGT-3’, antisense) was inserted into pSilencer according to the manufacturer's instructions, and it was transfected into DLD-1 and CCK-81 cells by the LipofectAMINE method (Life Technologies, Inc., Tokyo, Japan). Two stably transfected clones were selected after hygromycin (800 μg/mL) treatment and were used for subsequent experiments. Mock empty vector transfecants of each cell line were used as controls. Inhibition of FHIT expression in both CCK-81 and DLD-1 was confirmed by fluorescent staining with FHIT antibody. Inhibition of FHIT with FHIT siRNA in CCK-81 compared with FHIT-expressing CCK-81 was examined by Western blotting analysis.

**Western blot analysis.** Cells (5×10⁵/mL) were washed with PBS and lysed for 20 minutes in 2 mL of 25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100 at 4°C. The lysate was homogenized by passing the sample through a 22-gauge needle. In brief, for immunoblot analysis, the samples were subjected to SDS-PAGE in 15% acrylamide gels under reducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk and 0.05% Tween 20 in PBS, blots were incubated with COX-2 monoclonal antibody and/or FHIT polyclonal antisera. After several washings, blots were incubated for 1 hour with goat antimouse IgG (1:5,000) coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence (ECL) Western blotting kit (Amersham, Buckinghamshire, United Kingdom).

**ELISA and MTT assays for colorectal cancer cells with FHIT siRNA.** In both DLD-1 and CCK-81 cells, PGE₂ production in FHIT⁻ siRNA-treated cells was compared with the FHIT⁺ control cells by the ELISA assay described above. In addition, cell proliferation rate in FHIT⁻ siRNA-treated cells was compared with the rate for FHIT⁺ control cells using the MTT assay described above.

**Epidermal growth factor receptor transfection.** According to the results of the current immunohistochemical study, we disclosed concordant...
expression of COX-2 and FHIT proteins. We assumed that epidermal growth factor receptor (EGFR) on inflammatory lesions in colorectal cancer tumors is a key molecule because a recent study reported that tobacco smoking activates EGFR signaling, thereby contributing to the elevated levels of COX-2 found in the oral mucosa of smokers (17). We prepared EGFR vector pLSX (provided by Prof. A Takayanagi, Keio University). Subsequent to cutting 3.9-kb EGFR out at the XhoI site from the pLSX vector, EGFR was inserted into the pBKCMV-hEGFR vector (Stratagene, La Jolla, CA; ref. 18).

In our previous study, we discovered mutations of the EGFR gene in >10% of cases of sporadic colorectal cancer (19). Considering the practical role of EGFR on inflammatory lesions, we examined alteration of COX-2 and FHIT expression in EGFR-mutated clones as well as EGFR wild-type clones. In addition to the wild-type plasmid of EGFR, we established mutant clones that had been discovered in human cases of sporadic colorectal cancer in our previous study. We artificially altered nucleotides at 2245 G>A (E749K) in exon 19 and 2285 A>G (E762G) and 2299 G>A (A767T) in exon 20. To establish those mutant clones, we followed the manufacturer’s protocol of the QuickChange Site-Directed Mutagenesis kit (Stratagene). Those mutant plasmids were inserted into the expression vector, and subcloning was done. We considered the EGFR (wild-type)–inserted plasmid without expression of the gene as a “mock” clone.

For transfection, 6 x 10⁴ COLO205 cells were seeded in DMEM with 10% FCS on six-well culture plates. After 24 hours, medium was exchanged with 2 mL DMEM supplemented with 0.2% FCS and cultured for an additional 4 hours. Transfection was done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, with minor modifications. Briefly, 5 µg plasmid DNA purified by an endotoxin-free purification system (Qiagen, Tokyo, Japan) were diluted in 50 µL serum-free DMEM. The diluted DNA was mixed with 4 µL reagent, which was prediluted in 50 µL serum-free DMEM. The mixture of DNA and reagent was added to the culture medium in a dropwise manner. Two-milliliter DMEM with 20% FCS was added to the culture medium. Cells were grown for 48 hours and subjected to protein study.

Immunoblot. Cells with wild-type EGFR and mutant clone-transfected cells were harvested, washed with PBS, and homogenized in lysis buffer containing 1 mmol/L Tris-HCl, 2 mmol/L EDTA, 100 mmol/L NaCl, 1% NP40, 1% Triton X-100, 10 mmol/L sodium orthovanadate, 5% leupeptine.

### Table 1. Clinicopathologic characteristics of COX-2 or FHIT expression in colorectal cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>COX-2+ (n = 41)</th>
<th>COX-2- (n = 51)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>FHT- (n = 12)</td>
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<tr>
<td>Sex</td>
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<td>Female (n = 39)</td>
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<td>5</td>
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<tr>
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<td>Well differentiated (n = 38)</td>
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<td>Moderately differentiated (n = 47)</td>
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<td>Depth of invasion</td>
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<tr>
<td>Beyond subserosal layer (n = 49)</td>
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<td>11</td>
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<tr>
<td>Lymph node metastasis</td>
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</tr>
<tr>
<td>Negative (n = 51)</td>
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<td>6</td>
</tr>
<tr>
<td>Positive (n = 41)</td>
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<td>6</td>
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<tr>
<td>Lymph vessel permeation</td>
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<td>Negative (n = 50)</td>
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<td>Positive (n = 42)</td>
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<td>Positive (n = 27)</td>
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<tr>
<td>Duke’s stage</td>
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<td>A, B (n = 51)</td>
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<td>6</td>
</tr>
<tr>
<td>C, D (n = 41)</td>
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<td>6</td>
</tr>
</tbody>
</table>

Abbreviation: ns, not significant.

### Table 2. Relationship between FHIT and COX-2 protein expression

<table>
<thead>
<tr>
<th>COX-2 protein expression</th>
<th>Positive (n = 41)</th>
<th>Negative (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHIT protein expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (n = 43)</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>Negative (n = 49)</td>
<td>12</td>
<td>37</td>
</tr>
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NOTE: A significant association was observed between FHIT expression and COX-2 expression (P < 0.001).
and 5 mmol/L phenylmethylsulfonyl fluoride with complete proteinase inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan). After samples were centrifuged for 10 minutes at 9,000 g, inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan). After samples were centrifuged for 10 minutes at 9,000 g, inhibitors were electrotransferred to a polyvinylidene difluoride membrane (AMRESCO, Solon, OH). The membrane was blocked with 2.5% skim milk and 2.5% bovine serum albumin in TBS supplemented with 0.1% Tween 20, stained at 4°C for 2 hours with secondary antibodies (Amersham, Piscataway, NJ), and visualized on film by the ECL-plus system (Amersham). Expression of EGFR, FHIT, and COX-2 proteins were adjusted by actin expression and were calculated by NIH Image J. Expression of EGFR, FHIT, and COX-2 proteins were adjusted by actin expression and were calculated by NIH Image J.

Statistical evaluation. Associations among protein expression and clinicopathologic variables were computed using either the two-tailed χ² test or Fisher's exact test, as appropriate. A comparison of PGE₂ production by ELISA between FHIT-expressing and nonexpressing cells was calculated by Student's t test. Cell proliferation as evaluated by mean absorbance was compared between FHIT⁺ and FHIT⁻ cells, and the average at each elapsed time was also calculated by Student's t test. P < 0.05 was considered statistically significant. In Western blotting of EGFR transfectants, linear regression analysis was applied to the relationship between EGFR and COX-2 and the relationship between EGFR and FHIT.

Results

Immunohistochemical analysis of FHIT and COX-2 in colorectal cancer tumors. We found that COX-2 was overexpressed in cancerous and surrounding noncancerous cells in samples obtained from colorectal cancer resections, whereas adenoma samples showed substantial COX-2 expression in interstitial tissue (data not shown) consistent with a previous report (20). We evaluated COX-2 and FHIT expression in adenocarcinomas of the colon and rectum (Fig. 1). Analysis of

![Western blot of FHIT-inducible H460 transfectants after exposure to ponasterone (Pon) A as inducer. Left, after addition of ponasteron A (10 μmol/L) to the medium, FHIT-inducible transfectants with full-length FHIT cDNA in pIND vector and control transfectants with empty pIND vector were subjected to Western blot with anti-FHIT antiserum. Clear induction was observed in FHIT-inducible cells, whereas no expression was detected in the control. Right, Western blot was performed to assess COX-2 expression between FHIT-inducible cells (lanes 1, 3, and 5) and empty vector control cells (lanes 2 and 4) after exposure to 10 μmol/L ponasterone A for 24 hours. Increasing amounts of LPS were added to the medium and cultured for an additional 72 hours (final LPS concentration: lane 1, 0.1 μg/mL; lanes 2 and 3, 0.5 μg/mL; lanes 4 and 5, 1.0 μg/mL). The medium was exchanged every 24 hours with fresh medium containing those reagents. COX-2 expression was increased in an LPS dose-dependent manner, although not relevant with FHIT induction. B, ELISA assay of PGE₂ production. FHIT-inducible H460 cells [FHIT(+)] and control empty vector transfectants [FHIT(−)] were cultured in medium with ponasterone A for 24 hours and subjected to ELISA assay after stimulation with 1.0 μg/mL LPS (left), 10 ng/mL PMA (middle), and 10 ng/mL IL-1β (right). Four independent experiments were done to confirm reproducibility. Representative data. LPS stimulation led to an apparent difference between FHIT⁺ and FHIT⁻ cells under the test conditions. C, MTT cell proliferation assay (left) and protein study (right). FHIT-inducible H460 cells [FHIT(+)] and control empty vector transfectants [FHIT(−)] were cultured in medium with ponasterone A for 24 hours and cultured with addition of 1.0 μg/mL LPS for indicated time, then subjected to the MTT assay. Similarly, expression of FHIT and COX-2 was assessed by Western blot at indicated time. Increased COX-2 expression was detected, whereas alterations of FHIT expression were not apparent.
The clinicopathologic features of our sample of colorectal cancer cases revealed that COX-2+ tumors with FHIT+ expression showed more advanced malignant features. As shown in Table 1, among the COX-2+ cases, 11 of 12 (91.7%) cases of FHIT+ colorectal cancer invaded beyond the subserosal layer, whereas more than half of the 29 cases of FHIT+ colorectal cancer had invasion restricted within the muscular layer. Thus, depth of tumor invasion was more advanced in FHIT+/COX-2+ cases. In addition, among the 51 COX-2+ cases, disease stage was either Duke’s A or B in 12 of 14 (86%) FHIT+ cases; this means that these cases showed a relatively early stage of disease. Table 2 shows the relationship between FHIT and COX-2 expression; there is a significant association with each other ($P < 0.001$). Two thirds (29 of 43, 67%) of the FHIT+ cases were also COX-2+, whereas 37 of the 49 (76%) FHIT+ cases were COX-2+.

**COX-2 expression is not altered by FHIT expression.** As shown in Fig. 2A (left), induction of FHIT expression was successful. COX-2 expression increased in line with elevation in LPS concentration; however, expression of COX-2 was not affected by FHIT expression, which was regulated by ponasterone A (Fig. 2A, right). Expression of both genes was independent of the other.

**Overexpression of FHIT suppresses PGE2 synthesis.** PGE2 production was significantly inhibited in FHIT transfectants treated with ponasterone A compared with vector-only transfectant (FHIT−) cells treated with ponasterone A. Compared with FHIT− cells, exposure of FHIT+ cells to LPS, IL-1β, or PMA suppressed PGE2 production (Fig. 2B). Thus, the averages ± SE of PGE2 production after LPS stimulation in FHIT+ cells and FHIT− cells were 119.250 ± 33.09 and 216.39 ± 22.62, respectively. After PMA stimulation, averages were 152.26 ± 39.44 and 192.67 ± 67.37.
respectively. After IL-1β stimulation, the average ± SE of PGE_2 production in FHIT⁺ cells and FHIT⁻ cells was 187.17 ± 82.094 and 228.16 ± 103.74, respectively. With respect to the most appropriate COX-2 inducer, stimulation by LPS showed the largest difference between FHIT-expressing cells and FHIT-nonexpressing cells (Fig. 2B; P < 0.018).

Overexpression of FHIT suppresses PGE_2-stimulated cell proliferation. As shown in Fig. 2C (left), proliferation of both FHIT⁺ and FHIT⁻ cells increased in response to LPS stimulation. The most notable difference was observed at 96 hours after addition of a stimulator. At this time point, the average absorbance value obtained in 12 experimental data points between FHIT⁺ and FHIT⁻ cells was 1.244 ± 0.046 and 1.487 ± 0.056, respectively (P = 0.0027). At earlier times, the difference did not reach statistical significance; however, cell proliferation was generally lower in FHIT⁺ cells than in FHIT⁻ cells. In addition, it is notable that the difference in PGE_2 was remarkable between the two LPS-stimulated cells and the two non–LPS-stimulated cells in spite of FHIT status. We therefore concluded that cancer cells were affected by inflammatory stimulation. However, the difference was clearly observed in response level between FHIT⁺ and FHIT⁻ expression in LPS⁺ cells.

We added ponasterone A every 24 hours to maintain continuous expression of FHIT protein. According to Western blotting analysis (Fig. 3C, right), COX-2 expression followed by PGE_2 activation was activated by LPS stimulant, which was independent from FHIT expression. In addition, we confirmed that FHIT expression was not directly affected by LPS stimulation; therefore, we concluded that the difference in cell proliferation between FHIT⁺ and FHIT⁻ cells under LPS stimulation lies in the influence of LPS on PGE_2 synthesis.

FHIT-knockdown colorectal cancer cells show an up-regulation of PGE_2 synthesis. We used DLD-1 and CCK-81 as FHIT mRNA-expressing colorectal cancer cell lines to be treated with FHIT siRNA as assessed by real-time RT-PCR (Fig. 3A). Fluorescence staining of FHIT protein in CCK-81 as well as DLD-1 was reduced by FHIT siRNA treatment (Fig. 3B), and expression of FHIT protein in siRNA-treated CCK-81 cells was suppressed to 24% of the level seen in parent FHIT-expressing cells, as assessed by Western blotting analysis (Fig. 3C).

As shown in Fig. 4A, production of PGE_2 was remarkably different between FHIT⁺ control colorectal cancer cells and FHIT⁻ siRNA-treated colorectal cancer cells. Especially in CCK-81 cells, synthesis of PGE_2 was inhibited under FHIT expression until 96 hours, whereas it was distinctively activated from 24 to 96 hours by LPS stimulation, with statistical significance by Student’s t test (P < 0.05). Although the DLD-1 line had relatively higher production of PGE_2 in FHIT⁻ siRNA-treated cells than in FHIT⁺ control cells, there was no statistical significance between them.

FHIT-knockdown colorectal cancer cells proliferate with PGE_2 stimulation. As shown in Fig. 4B, the proliferation rate of CCK-81 cells was remarkably higher in FHIT-knockdown cells than in FHIT-expressing control cells, achieving statistical significance (P < 0.05) at 24, 48, 72, and 96 hours. On the other hand, DLD-1 showed no statistical significance in proliferation rates, but the cell proliferation rate in FHIT-knockdown cells was more than that in FHIT⁺ DLD-1 cells. The CCK-81 line showed a much larger
expression of FHIT expression. Wild-type and 2299/A767T mutant showed higher EGFR expression compared with other samples, concordant with increased expression of FHIT and COX-2.

Figure 5. Association of induced EGFR expression with COX-2 and FHIT expression. Wild-type and mutant EGFR cDNA vectors were transfected and subjected to Western blot for assessment of COX-2 and FHIT expression. Up-regulation of both COX-2 and FHIT expression in EGFR cDNA transfected colorectal cancer cells was observed without LPS stimulation. Actin expression indicates an equal amount of protein loaded. Bottom, relative ratios of each expression. Wild-type and 2299/A767T mutant showed higher EGFR expression compared with other samples, concordant with increased expression of FHIT and COX-2.

Expression of FHIT is activated by abundant expression of EGFR. Figure 5 (bottom) shows expression ratios in each clone, such as wild type, and in three mutants compared with expression in mock cells. A significant association was observed between EGFR expression and FHIT expression ($P = 0.01$, $R = 0.958$). EGFR expression and COX-2 expression also showed concordance with each other; this finding has already been reported by Moraitis et al. (17).

Discussion

Immunohistochemical study showed a significant relationship between FHIT and COX-2 expression in colorectal cancer tumor samples; there was a tendency toward positive COX-2 expression in FHIT+ tumors and vice versa. Our previous study of colorectal cancer reported that FHIT+ tumors showed more aggressive biological behavior than FHIT− tumors. Expression status of COX-2 has also been reported to correlate with aggressive colorectal cancer behavior (21). Taken together, colorectal cancer tumors with COX-2+ and FHIT− expression may be considered the most aggressive compared with the other three expression combination groups. Indeed, as shown in Table 1, deeper invasion was more frequently seen in COX-2+, FHIT− tumors than tumors with the other three expression combinations.

As mentioned above, there was a significant association between FHIT and COX-2 expression when studied with immunohistochemistry. In contrast, there was no direct interaction between FHIT and COX-2 proteins in in vitro study (Fig. 2A). We then focused on PGE2 because COX-2 is not thought to be directly associated with carcinogenesis; however, PGE2 is intimately involved in carcinogenesis and in cancer biology (22). PGE2 plays a main role in tumor cell proliferation through activation of molecules and pathways, such as EGFR, extracellular signal-regulated kinase 2 (23), and phosphatidylinositol 3-kinase signal pathways (24, 25). Moreover, PGE2 is related to inhibition of apoptosis (26), activation of angiogenesis (27), and promotion of invasion or metastasis (28).

In the current study, PGE2 production was significantly inhibited in enforced FHIT-expressing cells compared with vector-only transfectant (FHIT−) cells. Cellular proliferation of both FHIT+ and FHIT− cells increased in response to inflammatory stimulation. However, FHIT− cells showed reduced cell biology. On the other hand, FHIT-knockdown colorectal cancer cells showed increased production of PGE2 as well as increased cell proliferation rate.

Given concordant expression between FHIT and COX-2 using immunohistochemical study, we focused on EGFR as the common upstream molecule. Moraitis et al. have reported that tobacco smoke activates EGFR expression in oral mucosa, and that its signal contributes to elevated levels of COX-2 (17). In addition, Dannenberg et al. have described a relationship among EGFR, COX-2, and carcinogenesis (29). In the current study, as shown in Fig. 2, induction of COX-2 was affected by LPS stimulation but not by FHIT. Moreover, Fig. 5 shows concordant expression of FHIT protein and COX-2 protein along with various expression levels of EGFR protein in wild-type and/or mutant-type clones in the absence of LPS inflammatory stimulation. Therefore, we conclude that COX-2 and FHIT were elevated by EGFR expression as one of the common upstream molecules. However, it is not known how FHIT is activated by EGFR, a receptor-type tyrosine kinase.

Considering together the inverse correlation between FHIT and PGE2 and the positive association between FHIT and COX-2, we speculated that in colorectal cancer subjected to inflammatory stimulation, COX-2 expression is associated with abundant FHIT expression. In endogenously FHIT− colorectal cancer tumors, PGE2 production was suppressed, which led to regulation or quenching, if any, of cell proliferation activity downstream. However, FHIT− colorectal cancer, presumably due to genetic and epigenetic alterations, may have lost physiologic regulation of PGE2 production, which would contribute to malignant cellular behavior. FHIT is supposed to function as a tumor supressor, at least partially via regulation of PGE2 production, even in a COX-2− background.

In conclusion, an analysis of FHIT and COX-2 expression in colorectal cancer tumors would be helpful to clinicians by indicating malignant potential. One could select patients with altered FHIT expression as candidates for treatment with COX-2 inhibitors aimed at prevention of cancer progression. Moreover, abundant expression of FHIT protein should have a role as a cancer suppressor against inflammation-mediated carcinogens that are mainly regulated by PGE2.

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