Restored Expression of Fragile Histidine Triad Protein and Tumorigenicity of Cervical Carcinoma Cells

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**Background:** Allelic losses in the short arm of chromosome 3 are common in cervical carcinomas. The fragile histidine triad (FHIT) gene at chromosome region 3p14.2 is a candidate tumor suppressor gene that may play a role in cervical tumorigenesis. We and others have identified aberrant FHIT transcripts and frequent loss of Fhit protein expression in primary cervical cancers and high-grade noninvasive lesions but not in normal cervical tissues. The altered expression of FHIT may be due to somatic mutations or integration of human papillomavirus DNA at the FHIT locus. The purpose of this study was to determine whether ectopic expression of Fhit can suppress the tumorigenic properties of cervical cancer cells.

**Methods:** We employed infection with recombinant retroviruses as well as transfection of plasmid DNA to restore Fhit protein expression in cervical cancer cell lines lacking full-length FHIT transcripts and endogenous Fhit protein. The effects of Fhit expression on tumor cell morphology, anchorage-independent growth, and tumorigenicity in nude mice were examined.

**Results:** Stable overexpression of Fhit had no discernible effect on the tumorigenic properties of two cervical carcinoma cell lines or on a lung carcinoma cell line previously reported by others to be suppressed for tumorigenicity by Fhit.

**Conclusions:** Restoration of Fhit expression does not suppress anchorage-independent growth or tumorigenicity of cervical carcinoma cell lines. However, it remains possible that FHIT inactivation may be important early in cervical tumor progression or that FHIT may suppress tumorigenesis in ways distinct from those measured by the assays employed in this study. [J Natl Cancer Inst 2000;92:338–44]
Cells. All human carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cervical carcinoma-derived cell lines HeLa, SiHa, C-33A, and C-4II were cultured in Dulbecco's modified Eagle medium (DMEM)/10% (vol/vol) fetal bovine serum (FBS). ME-180 cervical carcinoma cells were cultured in McCoy's 5A medium containing 10% FBS. CaSki cervical carcinoma and H460 lung carcinoma cells were cultured in RPMI-1640 medium/10% FBS, and AGS gastric carcinoma cells were maintained in Ham's F12 nutrient medium/10% FBS. Media and FBS were obtained from Life Technologies, Inc. (GIBCO BRL), Gaithersburg, MD. Human primary foreskin keratinocytes were cultured from fresh neonatal foreskins and maintained in keratinocyte growth medium (Clonetics, Walkersville, MD), as previously described (25). Amphotropic Phoenix cells, which are highly transfectable 293T-derived human embryonic kidney cells stably expressing the MoLV retroviral gag, pol, and env genes, were used to package and amplify recombinant retroviruses ([26] and http://www.stanford.edu/group/nolan/nel-phoenix.html). These cells were maintained in DMEM/10% FBS.

Vector construction. Full-length, wild-type FHIT complementary DNA (cDNA) was inserted into the EcoRI site of the pP8GS–CMV–CITE–Neo(+) plasmid (provided by G. Nabel, University of Michigan, Ann Arbor), which contains the retroviral long terminal repeat and packaging signal for efficient expression. Chimeric transcripts encoding the gene of interest fused to the neomycin resistance gene were generated from this vector. An internal ribosomal entry site allows translation of both proteins from the chimeric transcript. The FHIT cDNA was also subcloned into the EcoRI site of pcDNA3.1/zeo+ vector (Invitrogen Corp., Calsin, UT) to generate pcDNA3.1/zeo+/FHIT, in which human embryonic kidney cells stably expressing the retroviruses we transfected with amphotropic Phoenix promoter. The full-length FHIT inserts of both plasmids were obtained by limiting dilution. Individual clones were initially evaluated for FHIT protein expression 3 months following transfection (passage 4) and again at passage 7. Cells were injected into nude mice at passage 7.

Western blot analysis. Lysates from cultured cells were prepared in lysis buffer composed of 50 mM 1% sodium dodecyl sulfate (SDS)/10 mM Tris (pH 7.4) and one tablet Complete™ protease inhibitor cocktail (Boehringer Mannheim Biochemicals). Xenograft tissues were homogenized in lysis buffer, and the supernatants were obtained by centrifugation at 13,000 g for 30 minutes at 4°C. Protein concentrations were measured by the bicinchoninic acid protein standard method (Pierce Chemical Co., Rockford, IL). Equivalent amounts (30–100 μg) of protein from each sample were loaded on 12% resolving polyacrylamide gels and then transferred to Immobilon-P membrane (Millipore Corp., Marlborough, MA). Fhit protein was detected with an anti-gest–Fhit rabbit polyclonal antibody (provided by K. Huebner) at a dilution of 1:2500 (27). Detection of antigen–antibody complexes was carried out with an enhanced chemiluminescence detection kit (Amer sham Life Science Inc., Arlington Heights, IL) per the manufacturer's protocol.

Southern blot analysis. To determine the independence of C-33A clonal sublines transfected with pcDNA3.1/zeo+/FHIT or pcDNA3.1/zeo+. Southern-blot analyses were performed. Genomic DNA was isolated from cultured cells by use of SDS/proteinase K digestion followed by phenol/chloroform extraction. Ten micrograms of each DNA sample was digested with 100 U of EcoRI at 37°C overnight. The digested DNA samples were separated by agarose gel electrophoresis, then transferred to Zeta probe membrane (Bio-Rad Laboratories, Inc., Hercules, CA). Hybridization to a 32P-labeled pcDNA3.1/FHIT probe was carried out in Rapid Hyb Solution (Amer sham Life Science Inc., Rockford, IL). Hybridized membranes were exposed to XAR5 autoradiography film (Kodak, Rochester, NY) for varying lengths of time at −80°C. Several clones revealing unique band patterns were selected for further analysis.

Colony formation in soft agar. Dishes (35 mm) were precoated with 1 mL of 0.6% agar/1x complete medium (medium with FBS). Suspensions of 104 cells in 0.3% agar/1x complete medium were spread over precoated wells and fed twice per week by adding small amounts of growth medium. Each experiment was performed in duplicate. Cultures were incubated for 4 weeks, then fixed and stained with 1.5% glutaraldehyde/0.06% methylene blue (vol/wt) solution. The total number of colonies in each dish greater than 100 μm in diameter was counted with the aid of a microscope eyepiece containing a grid.

Tumorigenicity in nude mice. Polyclonal populations of C-33A, C-4II, and H460 cells transfected by use of recombinant retroviruses with and without the FHIT cDNA inserted and several independent clones of C-33A cells stably transfected with pcDNA3.1/zeo+/FHIT or pcDNA3.1/zeo+ vector alone were evaluated for tumorogenicity in nude mice. Six-week-old female athymic nude (nu/nu) mice (Charles River Laboratories, Wilmington, MA) were given subcutaneous injections in the left and right flanks of 5 × 106 cells in 0.2-mL Hanks' balanced salt solution without phenol red (Life Technologies, Inc.). Cells transfected/transduced with each construct were injected into five mice, which were ear-tagged and followed individually throughout the study. Mice were examined twice to three times per week for tumor formation at the sites of injection. In situ tumor measurements were taken at least once per week starting on day 7. Experiments were terminated 30 days (C-33A and C4II) or 2 weeks (H460) after injection. H460 xenografts were harvested earlier, since previous experiments with the parental cells showed that growth for longer periods of time resulted in significant tumor necrosis. Animals were killed with CO2, then examined for tumor growth at the injection sites and for tumor dissemination. Tumors were weighed and measured with linear calipers, then divided into several portions for further analyses. Representative samples of each tumor were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Tissue sections (5 μm) from representative xenografts were cut and mounted on superfract Plus slides (Fisher, Isasca, IL) and stained with hematoxylin-eosin for histopathologic evaluation or used for immunohistochemistry. Representative portions of xenograft tissues were also snap-frozen in liquid nitrogen and stored at −80°C until utilized as a source of protein for western blot analysis. All animal studies were conducted in accordance with a study protocol approved by the University of Michigan’s Committee on Use and Care of Animals.

Immunofluorescent staining of Fhit protein. Approximately 105 cultured cells were seeded on 3-aminopropyltrithoxysilane-treated slides (Dige ne Diagnostics, Inc., Northfield, MN), grown for 2 days, fixed in 4% paraformaldehyde, then permeabilized in 1% goat serum/0.5% Triton X-100/phosphate-buffered saline (PBS). After washing with PBS, the slides were blocked with 20% goat serum for 30 minutes at room temperature. Samples were incubated with the rabbit polyclonal anti-Fhit antibody at 1:200 dilution for 1 hour at room temperature. Slides were washed three times in PBS, then incubated with Texas Red-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Lab Inc., West Grove, PA) at a dilution of 1:150 for 30 minutes at room temperature. The stained cells were visualized under a fluorescence microscope (Olympus, Melville, NY).

Immunohistochemical staining of Fhit protein. Tissue sections (5 μm) were dewaxed in xylene, then rehydrated through graded alcohols. Antigen retrieval was performed by boiling slides in citrate buffer (pH 6.0; Biogenex, San Ramon, CA) in a microwave oven for 10 minutes. Endogenous peroxidase activity was blocked by incubation with 6% hydrogen peroxide in methanol. Slides were then incubated with primary antibody (polyclonal rabbit gest-Fhit antiserum) at a dilution of 1:4000 over-
null was not detected by western blot analysis in either C-33A or C-4II cervical carcinoma cells (Fig. 1, A). Fhit protein of the expected size was detected in HeLa, CaSki, SiHa, and ME180 cervical cancer cells, as well as in primary foreskin keratinocytes (Fig. 1, A). Our detection of full-length FHIT transcripts and Fhit protein in HeLa contrasts with other studies in which Fhit protein was reported to be undetectable (18). The different observations of Fhit expression in HeLa likely reflect the genetic variability of HeLa sublines that have been used by different laboratories (20,27). C-33A and C-4II cell lines are tumorigenic in nude mice, forming poorly differentiated squamous carcinomas on subcutaneous injection into the flank. H460 cells, derived from a large-cell carcinoma of the lung, also form poorly differentiated tumors in nude mice. Previous studies (17,29) have shown that H460 cells harbor homozygous deletion of FHIT (introns 5) and lack expression of endogenous Fhit protein. Western blot analysis confirmed lack of Fhit protein expression in H460 cells (Fig. 1, B) and AGS cells (data not shown). In our studies, the injection of up to \(1 \times 10^7\) AGS gastric carcinoma cells failed to generate tumors in nude mice during a 9-week sur-

**RESULTS**

**C-33A, C-4II, AGS, and H460 Cells and Expression of Endogenous Fhit Protein**

We evaluated several cervical carcinoma cell lines by immunoblot analysis and confirmed the absence of endogenous Fhit protein expression in two cell lines, C-33A and C-4II, previously shown to lack full-length FHIT transcripts (19). C-33A has a homozygous deletion within FHT, including exon 5 (4), and C4I, a cell line derived from the same cancer specimen as C-4II, has also been reported to have a homozygous deletion within the FHIT locus (28). As expected, Fhit protein was not detected by western blot

**Fig. 1.** Immunoblot analysis of fragile histidine triad (Fhit) protein expression in selected cancer cell lines. **Panel A:** Expression of endogenous Fhit protein in human primary foreskin keratinocytes (PKF) and in six human cervical carcinoma-derived cell lines (C-4II, CaSki, HeLa, ME180, SiHa, and C-33A) (100 \(\mu\)g total protein was loaded in each lane). **Panel B:** Expression of Fhit in polyclonal populations of C-33A and C-4II cervical carcinoma cells and H460 lung carcinoma cells generated by stable transduction with recombinant retroviruses (30 \(\mu\)g total protein loaded in each lane). Total protein was isolated from cells stably transduced by FHT recombinant retroviruses (F) or retroviruses lacking the FHT insert (V). Fhit expression in preinjection cells (C) and derivative tumor xenografts (X) is shown. **Panel C:** Fhit expression in polyclonal (P) and clonal (CL) C33A cell populations generated by stable transfection with pcDNA3.1(zeo+) (V) and pcDNA3.1(zeo+)/FHIT (F) expression plasmids (amounts of total protein loaded are indicated). Fhit expression in preinjection cells from one control clone V(CL1) and three Fhit expressing clones F(CL1–3) is compared with Fhit expression in representative derivative xenografts F(CL2) and F(CL3).
veillance period. AGS cells were, therefore, excluded from further studies of Fhit’s ability to suppress their tumorigenic growth in nude mice but included in our analysis of Fhit’s effects on anchorage-independent growth.

Restoration of Fhit Expression in C-33A, C-4II, and H460 Cells

Recombinant retroviruses carrying the cloned FHIT cDNA as well as a G418 selectable marker were used to transduce C-33A, C-4II, and H460 cells. Fhit protein of the expected size was easily detected in polyclonal populations of cells infected with retrovirus carrying FHIT and in the derivative tumor xenografts (Fig. 1, B, lanes 1–6) but not in cells infected with a retrovirus lacking the FHIT cDNA insert (Fig. 1, B, lanes 7–9). Expression of Fhit protein was also verified in six of 14 zeocin-resistant clones of C-33A cells stably transfected with the pcDNA3.1/zeo+/FHIT expression plasmid—three representative clones [C-33A-F(CL1, CL2, and CL3)] shown in Fig. 1, C, lanes 4–6. Four of the six pcDNA clones, including the three shown in Fig. 1, C, were shown to be independent by Southern blot analysis (data not shown). Fhit protein expression was not detected in any of 12 C-33A clones transfected with the pcDNA3.1/zeo+ vector alone—representative clone [C-33A-V(CL-1)] shown in Fig. 1, C, lane 3. Expression of Fhit protein in C-33A, C-4II, and H460 cells transduced by recombinant retroviruses was also confirmed by immunofluorescence staining prior to further analysis (Fig. 2, A–C). Some heterogeneity of Fhit expression was noted, with 30%–40% of stably transfected cells expressing very high protein levels and the remainder expressing lower, but detectable, amounts of Fhit protein. Fhit expression was not detected by immunofluorescence staining in control (empty vector) C-33A, C-4II, or H460 cells (data not shown). No obvious changes in cellular morphology or growth kinetics were noted in cells expressing Fhit protein.

Restoration of Fhit Expression and Tumorigenicity of Cervical or H460 Lung Carcinoma Cells

The parental cell lines were subcutaneously injected into nude mice to confirm their tumorigenic growth properties. Injection of $5 \times 10^6$ cells from the C-33A and C-4II cervical cancer lines and the H460 lung cancer line resulted in formation of palpable tumors after 2 weeks. Stably selected polyclonal populations of each cell line infected either recombinant FHIT or control retroviruses were injected into groups of five mice, so that each population of cells was evaluated at 10 injection sites. A total of six independent, stably transfected C-33A clones
[three transfected with pcDNA 3.1/zeo+/FHIT (C-33A F1–3) and three transfected with vector alone (C-33A V1–3)] were also evaluated. Expression of Fhit failed to suppress tumor formation within the surveillance period in any of the polyclonal or clonal cell lines tested. The net weights and volumes of tumor tissues obtained immediately after excision from euthanized mice are shown in Fig. 3, A–D. There was no statistically significant difference in the weight or volume of tumors formed by polyclonal populations of retrovirally transduced C-33A, C-4II, or H460 cells expressing Fhit versus control (P > .05; mixed models; Fig. 3, A and B). Similarly, there was no statistically significant difference in the weight or volume of tumors formed by clonal populations of C33A cells transfected with the pcDNA3.1/zeo+/FHIT plasmid versus vector control (P > .05; mixed models; Fig. 3, C and D). The tumors formed by parental and vector-only transduced C-4II cells were rather small, with in vivo regression observed starting 2 weeks after injection. Tumor formation by C-4II cells was documented by microscopic examination of xenograft tissues, and no differences were noted between Fhit-positive and the Fhit-negative cells. Tumor xenografts formed by Fhit-expressing C-33A, C-4II, and H460 cells were shown to retain strong Fhit protein expression by both immunoblot and immunohistochemical analyses. Fig. 1, B (lanes 2, 4, and 6), shows retention of Fhit expression in xenografts derived from polyclonal populations of cells transduced by recombinant retroviruses. Fig. 1, C (lanes 7 and 8), shows retention of Fhit expression in xenografts derived from clonal populations of C-33A cells stably transduced with the pcDNA3.1/zeo+/FHIT plasmid. Immunohistochemical detection of Fhit protein expression in representative tumor xenografts is shown in Fig. 2, D–L. Panels D and E show Fhit expression in a xenograft derived from a clonal population of C-33A cells (C-33A-F1) stably transfected with the pcDNA3.1/zeo+/FHIT plasmid. Panels F and G show Fhit expression in a xenograft derived from polyclonal C-33A cells transduced with the pPGS-CMV-CITE−Neo(+)/FHIT recombinant retrovirus. Panels H and I show Fhit expression in retrovirally transduced C-4II and H460 cells, respectively. Roughly comparable heterogeneity of Fhit protein expression was noted in preinjection cells and xenografts, with the xenografts retaining a substantial number of cells strongly expressing Fhit. For comparison purposes, the absence of Fhit expression in representative xenografts formed by polyclonal populations of C-33A, C-4II, and H460 cells retrovirally transduced with vector alone is shown in panels J–L.

**Restoration of Fhit Expression and Colony Formation in Soft Agar**

As shown in Table 1, cells with and without Fhit expression were examined for alterations in anchorage-independent growth properties. C-4II and AGS cells showed no difference in the number or size of colonies formed by cells expressing Fhit versus vector control (P > .05). However, C-33A and H460 cells expressing Fhit formed more and larger colonies than cells transduced with vector alone (all P = .001). This result suggests that, under certain conditions, Fhit expression may actually increase the anchorage-independent growth properties of selected cancer cells. A similar observation was made by Siprashvili et al. (17), who noted that AGS and RC48 cells formed larger colonies compared with control cells.

**DISCUSSION**

Tumor suppressor genes are perhaps most clearly distinguished by their biallelic inactivation in cancer as a result of deletions, point mutations, or epigenetic modifications such as promoter methylation. In cervical carcinomas, inactivation of tumor suppressor gene products can also occur through interactions between the HPV-transforming proteins E6 and E7 and the cellular proteins p53 and pRB, respectively. Tumor suppressor genes can also be defined functionally as genes whose protein products inhibit the tumorigenic or metastatic properties of cells. Because FHIT does not entirely conform to the classical functional and genetic criteria defining tumor suppressor genes, it has been difficult to definitively prove or exclude it as a bona fide tumor suppressor. Our studies demonstrate that restoration of Fhit expression fails to suppress anchorage-independent growth or tumorigenicity of two cervical carcinoma cell lines in a xenograft model. Otterson et al. (18) similarly found that Fhit is unable to suppress the tumorigenic properties of HeLa, another cervical cancer-derived cell line.

Studies of Fhit’s ability to suppress tumorigenicity of cancer cells continue to yield conflicting results. Collectively, we and Otterson et al. have demonstrated Fhit’s inability to suppress tumorigenicity of three different cervical carcinoma cell lines (HeLa, C-33A, and C4II). If, as Otterson et al. have suggested, Fhit exerts its

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**Fig. 3. Weight and volume of tumor xenografts.** Weight (A) and volume (B) volume of tumor xenografts from polyclonal populations of C-33A, C-4II, and H460 cells transduced by fragile histidine triad (FHIT) recombinant versus control retroviruses. Weight (C) and volume (D) volume of tumor xenografts derived from clonal populations of C-33A cells transfected with pcDNA3.1/zeo+/FHIT (C-33A F1–3) or pcDNA3.1/zeo+ vector alone (C-33A V1–3).
Table 1. Colony formation in soft agar: human cervical carcinoma (C-4II and C-33A), lung carcinoma (H460), and gastric carcinoma (AGS) cells transduced with fragile histidine triad (FHIT) recombinant vector versus control retroviruses*

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<th>Cell line†</th>
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<th>201–300 µm</th>
<th>&gt;300 µm</th>
<th>P linear association</th>
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<td>2</td>
<td>0</td>
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*Sum of numbers of colonies larger than 100 µm were counted in duplicate wells. Data were analyzed by a Mantel–Haenszel chi-squared test (linear association) and a general chi-squared test (for >100 versus <100 analyses).

†Polyclonal G418-resistant lines transduced with a control retrovirus [pPGS–CMV–CITE–Neo(+)] or a retrovirus carrying a full-length FHIT complementary DNA [pPGS–CMV–CITE–Neo(+)/FHIT].

tumor-suppressive effect in a cell lineage-specific manner (18). Fhit may well suppress tumorigenicity of cell lines derived from lung, kidney, or digestive tract cancers but not those derived from cervical cancers. However, this explanation cannot account for the different results obtained by three independent groups studying H460 lung carcinoma cells. Genetic heterogeneity of H460 sublines utilized in different laboratories may be, in part, responsible for the different outcomes because genetic variation at the FHIT locus has been described in other cancer cell sublines such as HeLa (27,30). The contradictory results may also reflect differences in the level and degree of heterogeneity of exogenous Fhit expression obtained in recipient cells (such as H460) by use of various gene delivery systems. In our hands, heterogeneous Fhit expression was observed when either retroviral or plasmid vectors were employed to express exogenous Fhit protein, even when clonal sublines of cells were isolated. Other investigators may have achieved more uniformly elevated levels of Fhit expression in the cells that they tested. However, if heterogeneity of Fhit expression is solely responsible for the different outcomes, one might expect a delay of tumor growth of Fhit-positive versus Fhit-negative control cells in addition to more uniform selection against Fhit-expressing cells in the xenografts. We made neither of these observations. Direct comparison of Fhit expression and function in cells generated by the different laboratories and with different constructs may well shed additional light on these problematic results. Notably, we found comparable levels of Fhit expression in clonal preinjection cells and their derivative xenografts, regardless of whether the cells initially expressed high [e.g., C-33A-F(CL2)] versus low [C-33A-F(CL3)] levels of Fhit protein (Fig. 1, C).

Although our study fails to provide additional functional evidence supporting FHIT as a tumor suppressor gene, we wish to emphasize that our analysis by no means excludes FHIT as a suppressor gene candidate. In light of the presence of frequent 3p14 deletions, FHIT somatic mutations, and alterations of FHIT expression in cervical cancer precursor lesions, it is possible that FHIT inactivation may be critical early in cervical cancer development or progression. However, restoration of Fhit expression in cervical cancer cells that have accumulated numerous other genetic alterations may not be sufficient for demonstrably affecting their growth or tumorigenic properties. Clearly, additional studies will be required to fully understand the role of FHIT in human cancer.

**References**


NOTES

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