Restoration of Fragile Histidine Triad (FHIT) Expression Induces Apoptosis and Suppresses Tumorigenicity in Breast Cancer Cell Lines

Cinzia Sevignani, George A. Calin, Rossano Cesari, Manuela Sarti, Hideshi Ishii, Sai Yendamuri, Andrea Vecchione, Francesco Trapasso, and Carlo M. Croce

Kimmel Cancer Center, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract

The fragile histidine triad (FHIT) gene at chromosome 3p14.2 is a tumor suppressor gene that is altered mainly by deletion in a large fraction of human tumors, including breast cancers. To evaluate the potential of FHIT gene therapy in this type of cancer, we have studied the biological effects of adenoviral FHIT transduction (Ad-FHIT) in breast cancer cell lines. The results showed that, after FHIT restoration in BT-549, MDA-MB-436, and HCC1806 cells, they underwent apoptosis by activation of the intrinsic pathway. In all three cell lines infected with Ad-FHIT, we have found activation of caspase-2, which is required for permeabilization of mitochondria, release of cytochrome c, and apoptosis. Furthermore, Fhit overexpression produces alteration in cell cycling properties, as well as reduction of the tumorigenic potential in nude mice.

Introduction

Breast cancer is among the most common human cancers, representing 32% of all cancers in the United States, and it is the leading cause of death for American women between ages 50 and 55 years (1). Despite important advances in chemotherapy, radiotherapy, and surgery, approximately 30% of patients with breast cancer will relapse and die of the disease. Therefore, complementary therapeutic strategies should be considered for improving the outcome of breast cancer patients. The FHIT gene, located at chromosome 3p14.2, encompasses the FRA3B and is a tumor suppressor gene involved in different epithelial cancers (2). FHIT gene deletions have been observed in several breast cancer cell lines and primary tumors, as well as in benign proliferative breast disease (3, 4). Furthermore, about 20% of primary breast carcinomas were reported to exhibit altered FHIT transcripts (4, 5). It was reported that in MDA-MB-231 cells, a mixture of normal and aberrant FHIT transcripts was present, whereas in MDA-MB-436 cells, no normal FHIT transcript was found (4). Interestingly, Fhit protein expression was reduced in approximately 50% of breast cancer samples, and the deletion frequency was higher in BRCA2- and BRCA1-linked breast carcinomas, consistent with the idea that loss of BRCA2 and BRCA1 function affects the stability of the FHIT/FRA3B locus (6, 7). Thus, genetic alterations at the FHIT locus were detected in a significant fraction of sporadic and familial breast cancers, suggesting an important role of this gene in breast tumorigenesis.

Several studies have reported that Fhit protein overexpression alters the cell cycle profile of malignant cells and triggers apoptosis. It was shown that transfection of FHIT in H460 lung cells was correlated with a high rate of apoptosis-induced DNA strand breaks, as well as with a significant G0-G1 arrest (8). More recently, virus-mediated Fhit gene transfer has been shown to induce apoptosis and reduce tumor growth in epithelial cancer cells from esophageal (9), lung (10, 11), cervical (11), and pancreatic cancers (12). A recent survey of the literature showed that 60% of primary tumors demonstrate absent or markedly reduced Fhit expression (13). Here, we investigated the effects of Fhit protein reexpression in Fhit-negative mammary tumor cells or in mammary tumor cells with low Fhit expression through an adenoviral vector. Modifications in cell proliferation, cell cycle profiles, and tumorigenicity are described. We observed that Ad-FHIT-transduced cells undergo caspase-mediated apoptosis; furthermore, they show impaired cell proliferation in vitro and a reduced ability to form tumors in nude mice.

Materials and Methods

Cell Culture. The breast cancer cell lines MDA-MB-436, MDA-MB-231, MDA-MB-468, BT-549, BT-747, SKBr3, MCF-7, HCC-70, HCC-1143, HCC-1395, HCC-1428, HCC-1569, HCC1806, HCC-1937, and HCC-1954 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 with 10% fetal bovine serum. 293 cells were purchased from Microbiix (Toronto, Ontario, Canada), cultured in DMEM and 10% fetal bovine serum, and used for packaging, expanding, and titration of Ad vectors.

Recombinant Adenoviral Vector and In Vitro Transduction. The Ad-FHIT vector was prepared according to the manufacturer’s instructions (Qbiogene, Carlsbad, CA) as described previously (9). An Ad-GFP vector was used as a control (Qbiogene). Viral vectors were amplified in 293 cells, and viral titers were determined by assay for PFUs. Cells (1 × 10⁶ cells/well for 6-well plates, 5 × 10⁵ cells/well for 12-well plates, and 1 × 10⁵ cells/well for 48-well plates) were transduced with Ad vectors by directly applying the diluted viruses into the growth medium at different MOIs. The transduction efficiency was determined by direct visualization using fluorescent microscopy of GFP-expressing cells after Ad-GFP infection. The transgene expression was detected by anti-GFP and anti-Fhit antisera by Western blotting.

Immunoblot Analysis. Immunoblot analysis was performed by standard protocols (14). Briefly, 2 × 10⁵ cells were cultured in 60-mm diameter dishes and lysed on ice in 300 µl of lysis buffer. Protein lysates were loaded on 4–20% linear gradient SDS-PAGE gels and electrophobted to polyvinylindene fluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with 5% skim milk and incubated with the indicated antisera. After reaction with an appropriate secondary antibody, the immunoreactive bands were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech), according to the manufacturer’s protocol. For Western blot analysis, we used the following antisera and concentrations: rabbit polyclonal anti-Fhit at 1:1000 (Zymed, South San Francisco, CA); mouse monoclonal anti-caspase-2 at 1:1000 (BD Biosciences, San Jose, CA); rabbit polyclonal anti-caspase-8 at 1:500 (Chemicon, Temecula, CA); rabbit polyclonal anti-caspase-9 at 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-PARP at 1:5000 (BD Biosciences); and anti-actin at 1:3000 (Sigma Chemical Co., St. Louis, MO).

Cell Growth and Flow Cytometry. For cell survival analysis, 1 × 10⁴ cells (MDA-MB-436, BT-459, and HCC1806) were seeded in 6-well plates, infected with Ad-FHIT and Ad-GFP at different MOIs, and counted in triplicate after 1 week. For growth curves, 1 × 10⁴ cells were cultured in 60-mm Petri dishes, infected using appropriate MOIs for each of the three breast cancer cell lines analyzed, and counted every day for 1 week. The number of viable cells/well was determined at the indicated times in triplicate, excluding...
tumors were processed for histology and assessed for human Fhit expression. Dako, Carpinteria, CA).

peroxide, and nonspecific binding sites were blocked with normal goat dilution; Zymed). Endogenous peroxidase was inhibited with 3% hydrogen injection was detected using rabbit antihuman Fhit antiserum (1:1000

vector was also injected intratumorally (H11003/ H11011). Ad-infected, Ad-FHIT-infected, and Ad-GFP-infected

were weighed after necropsy at the end of each experiment. The Ad-infection group. The tumor diameters were monitored weekly, and all tumors group were treated with infected cells, and 10 mice were treated for the mock injections of Ad-GFP (H11003/ H11011).

of 5-week-old female nu/nu mice with MDA-MB-436 and HCC1806 breast cancer cells. The control group received intratumoral injection, 5

mo (Charles River, Cambridge, MA); 5 mice per group were treated with infected cells, and 10 mice were treated for the mock infection group. The tumor diameters were monitored weekly, and all tumors were weighed after necropsy at the end of each experiment. The Ad-FHIT vector was also injected intratumorally (~3 × 10^6 PFU/tumor) into 1-cm tumors derived from s.c. inoculation of nu/nu mice with MDA-MB-436 and HCC1806 breast cancer cells. The control group received intratumoral injections of Ad-GFP (~3 × 10^6 PFU/tumor). Mice were euthanized after 48 h, and tumors were processed for histology and assessed for human Fhit expression.

Immunohistochemistry. Expression of Fhit protein after intratumoral injection was detected using rabbit antihuman Fhit antiserum (1:1000 dilution; Zymed). Endogenous peroxidase was inhibited with 3% hydrogen peroxide, and nonspecific binding sites were blocked with normal goat serum. After incubation with an appropriate biotinylated antiserum, slides were incubated with streptavidin horseradish peroxidase (1:1000 dilution; Dako, Carpinteria, CA).

the dead cells by trypan blue staining. Flow cytometry analysis was performed by standard protocols (14). Briefly, 2 × 10^4 cells were seeded in 100-mm dishes. Infections were at MOI 50, 150, and 300 on MDA-MB-436 cells; MOI 10, 50, and 100 on BT-549 cells; and MOI 50, 100, and 200 on HCC1806 cells. Cells were harvested after 48, 72, 96, 120, and 144 h; washed in PBS; and stained with propidium iodide (50 μg/ml). The samples were examined by flow cytometry on an EPICS-XL scan (Beckman-Coulter, Fullerton, CA) using doublet discrimination gating. The apoptotic fraction was also analyzed using the Active Caspase-3 PE Mab Apoptosis Kit (PharMingen, BD Biosciences, Mohrsville, PA), following the manufacturer’s protocol.

In Vivo Tumorigenicity and Intratumoral Injection of Ad-FHIT. Animal studies were performed according to institutional guidelines. The MDA-MB-436 and HCC1806 breast cancer cell lines were plated and infected in vitro under three conditions (Ad-FHIT, Ad-GFP, and mock infection) at MOI 350 for MDA-MB-436 and MOI 150 for HCC1806. Thirty-six h after infection, 5 × 10^4 cells were injected s.c. (1.5 × 10^6 cells/150 μl) into the left flank of 5-week-old female nu/nu mice (Charles River, Cambridge, MA); 5 mice per group were treated with infected cells, and 10 mice were treated for the mock infection group. The tumor diameters were monitored weekly, and all tumors were weighed after necropsy at the end of each experiment. The Ad-FHIT vector was also injected intratumorally (~3 × 10^6 PFU/tumor) into 1-cm tumors derived from s.c. inoculation of nu/nu mice with MDA-MR-436 and HCC1806 breast cancer cells. The control group received intratumoral injections of Ad-GFP (~3 × 10^6 PFU/tumor). Mice were euthanized after 48 h, and tumors were processed for histology and assessed for human Fhit expression.

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Statistics. Both the in vivo and in vitro results were expressed as mean ± SD or mean ± SE. Student’s two-sided t test was used to compare the values of the test and control samples. A value of P < 0.05 was taken as significant.

Results

Ad-transduced FHIT Expression in Breast Cancer Cells in Vitro. Analysis of endogenous Fhit protein expression on 15 breast cancer cell lines showed that endogenous Fhit protein was undetectable in MDA-MB-436, HCC-1395, and HCC1806 cell lines. All other breast cell lines expressed endogenous Fhit protein, but the levels of expression were reduced in BT-549, HCC-70, and HCC-1143 cell lines compared with normal breast cells (Clonetics, Walkersville, MD; data not shown). For additional experiments, we selected cell lines with undetectable (MDA-MB-436 and HCC1806) or low (BT-549) Fhit protein expression. Breast cancer cell lines were infected with either Ad-FHIT or Ad-GFP. Immunoblot analysis 48 h after infection showed FHIT transgene overexpression in all Ad-FHIT-transduced cells (data not shown). The transduction efficiency decreased in the following order: BT-549 > HCC1806 > HCC-1395 > MDA-MB-436. The transduction levels were as follows: (a) BT-549, about 30% at MOI 10, 50% at MOI 50, and 90% at MOI 100; (b) MDA-MB-436, about 30% at MOI 50, 60% at MOI 150, and 95% at MOI 300; and (c) HCC-1806, about 25% at MOI 50, 45% at MOI 100, and 90% at MOI 200.

Cell Growth Analysis of Adenoviral FHIT-infected Breast Cancer Cells in Vitro. For cell growth analysis, 1 × 10^4 cells (MDA-MB-436, BT-549, and HCC1806) were seeded in 6-well plates, infected with Ad-FHIT and Ad-GFP at different MOIs, and counted after 1 week. Cell counts showed a significant inhibition of cell growth in Ad-FHIT-transduced cells (Fig. 1, left panels). For growth kinetics curves,
1 x 10^5 cells were cultured in 60-mm Petri dishes and infected using appropriate MOIs. Cell counts revealed that in vitro growth of Ad-FHIT-infected cells (MDA-MB-436, BT-459, and HCC1806) was significantly inhibited compared with that of Ad-GFP-infected cells (Fig. 1, right panels). The growth inhibition of MDA-MB-436 was less pronounced than BT-549 and HCC1806 cells, presumably due to the lower rate of infection by Ads at MOI 10, 50, and 100.

**Cell Cycle Analysis and Apoptosis of Ad-FHIT-infected Breast Cancer Cell Lines.** To study cell cycle alterations induced by Fhit overexpression, each cell line was infected at several MOIs with either Ad-FHIT or Ad-GFP. A sub-G1 cell population was observed after Ad-FHIT transduction in all three cell lines, whereas no modifications in cell cycle profile were detected after infection with Ad-GFP. Ninety-six h after infection with Ad-FHIT (MOI 100), 12% of BT-459 cells were in the sub-G1 fraction, whereas 144 h after infection (MOI 200 and MOI 400, respectively), 17.8% of HCC1806 cells and 19% of MDA-MB-436 cells were dead (Fig. 2). For all of the cell lines tested, Fhit induction of cell death was MOI and time dependent (data not shown); moreover, MDA-MB-436 cells showed a marked G2-M transition block with massive cell death. Therefore, an early effect of Fhit reexpression in this cell line could be the induction of a G2 block, with subsequent activation of the cell death program.

To further assess the Fhit-dependent apoptotic program, we performed a caspase-3 activity test after infection with Ad-FHIT and Ad-GFP under the experimental conditions used for the cell cycle analysis. After infection with Ad-FHIT, the percentage of cells positive for the active form of caspase-3 was 18.4%, 22.3%, and 86.0% for BT-549, HCC1806, and MDA-MB-436, respectively, versus 8.95%, 8.96%, and 13.3% of the same cells infected with Ad-GFP.

**Analysis of the Apoptotic Pathways in FHIT-reexpressing Cells.** MDA-MB-436, BT-549, and HCC1806 breast cancer cell lines were infected with increasing MOIs, and the fraction of transduced cells was monitored by confocal microscopy and fluorescence-activated cell-sorting analysis. To exclude virus-specific effects, we compared cell lysates from cells infected with Ad-GFP at the same MOI. Statistically significant differences were observed in cell growth for Ad-FHIT and Ad-GFP infection at a range of MOIs in all three cell lines (Fig. 1). Further analyses used MOIs for which the largest effect was found.

To investigate the molecular mechanism of the oncosuppressive activity of Ad-FHIT, we assessed the expression of molecules involved in the apoptotic cascade, such as caspase-2, caspase-8, caspase-9, and PARP. In the MDA-MB-436 cell line, the caspase-2, caspase-9, and PARP levels were reduced in Ad-FHIT-infected cells with respect to Ad-GFP-infected cells, whereas caspase-8 was unaffected. Caspase-2 was also activated in both BT-549 and HCC1806 breast cancer cell lines, whereas caspase-8, caspase-9, and PARP levels were not significantly affected by overexpression of Fhit in the same cells. Variation in caspases and PARP levels between uninfected and Ad-GFP-infected cells was not detected (Fig. 3).

**Tumorigenicity of Ad-infected Breast Cancer Cell Lines in Vivo.** When nude mice were s.c. inoculated with BT-549, MDA-MB-436, and HCC1806 cells, the first two cell lines were tumorigenic, whereas the last one did not produce tumors, in concordance with previously published information about the characteristics of these breast cancer cell lines (15). Thus, we tested the tumorigenic potential of Ad-FHIT-infected MDA-MB-436 and HCC1806 cells in two groups of mice. In the first group, mice were inoculated with 5 x 10^5 MDA-MB-436 viable cells that had been infected in vitro at MOI 350 with Ad-FHIT (5 mice) or with
Ad-GFP (5 mice) or were mock-infected (10 mice) and cultured for 36 h before injection. Tumor growth was completely suppressed in mice inoculated with Ad-FHIT-infected MDA-MB-436 cells, whereas the average tumor weights for controls at the end of the experiment (Ad-GFP and uninfected wild-type cells) were 0.7 and 0.76 g, respectively (Fig. 4). In the second group, 5 mice were inoculated with 5 × 10⁶ HCC1806 viable cells that had been infected at MOI 150 with Ad-FHIT or with Ad-GFP (5 mice) or mock-infected (10 mice) and cultured for 36 h before s.c. injections in nude mice (bottom right). As controls, we used wild-type (uninfected) cells and Ad-GFP-infected cells.

To investigate the virus transduction efficiency in vivo, mice that received intratumoral injection were sacrificed 36–48 h after injection. Sections of tumors were analyzed for human Fhit protein expression by immunohistochemistry. Only tumor sections of mice treated with the therapeutic virus showed Fhit protein expression, which confirms the delivery of the transgene at the cellular level in vivo. The efficiency of transduction after intratumoral injection of Ad-FHIT was approximately 30% (data not shown).

**Discussion**

To confirm a role of FHIT in breast cancer development and the possible role of FHIT gene therapy for mammary cancer treatment, we evaluated the effect of viral transfer of the human FHIT gene into human breast cancer cells. Fourteen of 16 breast cancer-derived cell lines tested showed absence or reduced levels of Fhit protein, consistent with reports of Fhit protein loss in breast cancer (16, 17). Three breast cancer cell lines that are Fhit negative (MDA-MB-436 and HCC1806) or express a low level of Fhit (BT-549) were further analyzed. Infection with Ad-FHIT virus reduces cell growth of all three cell lines analyzed, and Ad-mediated Fhit overexpression induced apoptosis in the three breast cancer lines. Similar findings for Ad-FHIT infection of other types of cancer-derived cell lines, includ-
ing pancreatic (12), esophageal (9), lung (8, 11), and cervical cancer cell lines (11), have been reported, suggesting that FHIT gene therapy may be useful for treatment of various human cancers, including breast cancers.

This study shows evidence of efficient suppression of in vivo tumorigenicity of breast cancer cell lines by an Ad transducing the FHIT gene. A possible role for Fhit in gene therapy is supported by results of experiments after injection of nude mice with FHIT-transduced breast cancer cells. The tumorigenicity of MDA-MB-436 cells was completely suppressed by Ad-FHIT treatment, and a significant reduction in tumor size was observed in animals injected with FHIT-transduced HCC1806 cells.

The mechanism of tumor suppression by Fhit is not fully understood. Several studies have presented data showing that Fhit overexpression leads to activation of the two main proapoptotic caspase cascades, mitochondrial-mediated caspase-3 activation through caspase-9 (intrinsic pathway) and caspase-3 activation by caspase-8 [extrinsic pathway (9, 11, 12)]. Although we observed activation of caspase-3 in all three cell lines analyzed, only in MDA-MB-436 was activation of caspase-9 observed, and clear evidence of caspase-8 activation was not observed. Therefore, it seems that the apoptotic effect of Fhit in MDA-MB-436 is through mitochondrial-mediated caspase-3 activation by caspase-9, whereas in BT-549 and HCC1806 breast cancer cell lines, caspase-3 activation seems to be independent of caspase-9 and caspase-8. As reported previously in MCF-7 cells, in which the CASP3 gene is functionally deleted, caspase-9 is not always an obligatory upstream activator of caspase-3 (18). In line with this idea, we have found evidence for the activation of caspase-2 in the cell lines studied. This, to our knowledge, is the first report of activation of caspase-2 after FHIT overexpression in human cancer cell lines.

Despite the fact that it was the first mammalian apoptotic caspase to be identified (19, 20), caspase-2 has been considered a secondary player in apoptosis pathways. Recently, it was shown that caspase-2 is activated early in apoptosis and may occur upstream of mitochondrial permeabilization, as reported in a new model for the apoptotic intrinsic pathways (19, 21). Caspase-2 can regulate mitochondrial permeability, followed by caspase-9 activation or by activation of apoptotic effectors downstream of mitochondria. The three breast cancer cell lines used in this study show a marked decrease in pro-caspase-2 expression, suggesting that apoptosis after Ad-FHIT infection in these cell lines is mediated by activated caspase-2.

With regard to FHIT and TP53 status, all three breast cancer cell lines selected for this study lack p53 wild-type expression, suggesting that the apoptotic effect of Fhit is independent of the TP53 tumor suppressor gene. This is in line with the published data on lung carcinoma NCI H460 cells (11). Two of these cell lines analyzed in this study (MDA-MB-436 and HCC1806) did not show expression of Fhit protein, whereas BT-549 showed weak expression. The apoptotic fraction was higher in cell lines completely lacking Fhit protein, suggesting that the mechanism that leads to apoptosis in these breast cancer cell lines is dependent on Fhit status, as was reported for the apoptotic effect of Fhit in pancreatic cancer cell lines (12). Similarly, it has been shown that Ad-FHIT is able to induce apoptosis in lung cancer cells with loss of Fhit expression (10). The precise mechanism(s) that leads to apoptosis after exogenous Fhit expression remains to be elucidated.

A G2-M block associated with apoptosis in some cancer cell lines (MDA-MB-436 breast cell line, H460 lung cancer cell line, and TE12 esophageal cancer cell line) has been observed (9, 11). The molecular basis for this cell cycle effect is not known, but it suggests that an early effect of Fhit reexpression could be induction of a G2 block, with subsequent activation of the apoptotic program.

In conclusion, efficient induction of apoptosis in vitro and suppression of tumorigenicity in vivo of breast cancer cells by an adenoviral transduction of the FHIT gene suggests the future possibility of its use in gene therapy against this disease.

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References


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