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Induction of apoptosis by tumor suppressor FHIT via death receptor signaling pathway in human lung cancer cells

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Abstract

FHIT is a novel tumor suppressor gene located at human chromosome 3p14.2. Restoration of wild-type FHIT in 3p14.2-deficient human lung cancer cells inhibits cell growth and induces apoptosis. In this study, we analyzed potential upstream/downstream molecular targets of the FHIT protein and found that FHIT specifically targeted and regulated death receptor (DR) genes in human non-small-cell lung cancer (NSCLC) cells. Exogenous expression of FHIT by a recombinant adenoviral vector (Ad)-mediated gene transfer upregulated expression of DR genes. Treatment with a recombinant TRAIL protein, a DR-specific ligand, in Ad-FHIT-transduced NSCLC cells considerably enhanced FHIT-induced apoptosis, further demonstrating the involvement of DRs in FHIT-induced apoptosis. Moreover, we also found that FHIT targeted downstream of the DR-mediated signaling pathway. FHIT overexpression disrupted mitochondrial membrane integrity and activated multiple pro-apoptotic proteins in NSCLC cell. These results suggest that FHIT induces apoptosis through a sequential activation of DR-mediated pro-apoptotic signaling pathways in human NSCLC cells.

Keywords: FHIT; Tumor suppressor; Apoptosis; Death receptor; Caspase; Lung cancer

Lung cancer is the leading cause of cancer-related deaths. The pathogenesis of lung cancer involves a multistep process of genetic and molecular changes. Studies have shown that a genomic aberration in human chromosome 3p is the most frequent and earliest genetic event in lung tumorigenesis and that it affects several tumor suppressor genes and oncogenes in this region [1–3]. Researchers have identified the fragile histidine triad (*FHIT*) gene at chromosome 3p14.2 and characterized it as a tumor suppressor gene [1–3]. This gene is very vulnerable to environmental carcinogens and is frequently involved in allele loss, genomic rearrangement, and cytogenetic abnormalities in human cancer [4]. The FHIT protein consists of 147 amino acids and is a member of the histidine triad nucleotide-binding protein superfamily. Inactivation of the FHIT protein plays an important role in the development of several human cancers, including lung cancer [5–7]. Several lines of *in vitro* and *in vivo* experimental evidence have demonstrated the tumor suppression function of the *FHIT* gene [8–12]. Exogenous expression of the *FHIT* gene in 3p14.2-deficient cancer cells induces apoptosis and alters cell cycle kinetics in various types of cancer cells [13–15]. Also, a previous study implicated the FHIT-induced tumor suppression in FHIT-mediated inactivation of MDM2 and subsequent stabilization of p53 protein in human lung cancer cells [9]. Although the proapoptotic function of FHIT is well documented, the precise molecular mechanism by which FHIT exerts potent tumor suppression activity remains largely unclear. We hypothesized that FHIT specifically targets and activates certain cellular proteins involved in the apoptosis signaling pathway. In this study, we investigated cellular responses to exogenous expression of wild-type (wt) FHIT and explored the molecular mechanism involved in the FHIT-induced apoptotic pathway in human non-small cell lung cancer (NSCLC) cells transduced with a recombinant adenoviral vector carrying the

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FHIT gene (Ad-*FHIT*). Using a sensitive and quantitative multiprobe ribonuclease protection assay (RPA) with gene-specific probes for the most important factors involved in cell proliferation, apoptosis, and cell cycle regulation, we identified potential upstream and downstream molecular targets of the FHIT protein and found that FHIT specifically targeted and upregulated death receptors (DRs). Overexpression of FHIT in Ad-FHIT-transduced NSCLC cells considerably upregulated the expression of DR3, DR4, and DR5. We also showed that FHIT overexpression activated downstream of the DR-mediated apoptotic pathway, disrupting mitochondrial membrane integrity and activating caspase signaling pathway. Therefore, our study indicated that the mechanism by which FHIT induces apoptosis is mediated by activation of the DR and caspase cascade signaling pathways in human lung cancer cells. Our findings also provide insight into the molecular mechanism of FHIT in tumor suppression and suggest novel gene therapy strategies for lung cancer.

Materials and methods

Cell lines and recombinant adenoviral vectors. The human NSCLC cell lines H1299 and A549 were purchased from the American Type Culture Collection (Manassas, VA). H1299 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). A549 was maintained in Ham's F-12 medium with 10% FBS. The construction and production of Ad-*FHIT*, an adenoviral vector carrying the *p53* gene (Ad-*p53*), and an empty adenoviral vector (Ad-EV) were described previously [9].

Multiprobe ribonuclease protection assay (RPA). RPA was performed according to the manufacturer's instructions (Pharmingen, San Diego, CA). Briefly, total RNA was isolated from A549 and H1299 cells transduced with Ad-*FHIT*, Ad-*p53*, or Ad-*EV* or treated with phosphate-buffered saline (PBS) with TRIzol reagent (Life Technologies, Grand Island, NY). A multiprobe template set containing human DR genes and their effectors, including caspase 8 (Casp 8), FASL, FAS, DCR1, DR3, DR4, DR5, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), TNF receptor (TNFR), TNFR-associated death domain (TRADD), RIP, and L32, was used for T7 polymerase-directed synthesis of a high-specific-activity, ^{32}P-labeled antisense RNA probe set. The radiolabeled probe set was hybridized in excess to the target RNA in solution (10–20 μg) at 56 °C for 16 h. After hybridization, free probes and unprotected single-stranded RNAs were digested with RNases. The remaining "RNase-protected" RNA fragments were purified and resolved on a 5% denaturing polyacrylamide gel. The RPA images and intensity of the bands for the selected genes were analyzed using phosphorimaging with a Storm 860 analysis system (Molecular Dynamics, Sunnyvale, CA). The expression of the selected genes was quantified as the percentage relative to that of the housekeeping gene GAPDH.

Apoptosis analysis. Induction of apoptosis in NSCLC cells was analyzed using a fluorescence-activated cell sorter (FACS) with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, cells were plated in six-well plates (1×10^6 cells/well); 24 h later, they were transduced with Ad-*FHIT*, Ad-*p53*, or Ad-EV and treated with a recombinant TRAIL protein (R&D Systems, Minneapolis, MN) at a final concentration of 10 ng/ml. PBS was used as a mock control. At designated times after transduction, cells were harvested and apoptosis were analyzed according to the manufacturer's instructions (Roche Molecular Biochemicals).

Analysis of mitochondrial membrane potential. Changes in mitochondrial membrane potential in adenoviral vector-transduced cells were measured using flow cytometry with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining (Molecular Probes, Eugene, OR). JC-1 exists as a monomer at low concentrations or at low membrane potential and emits green florescence at 527 nm. However, at higher concentrations or higher membrane potential, JC-1 forms J-aggregates and emits red fluorescence near its emission maximum of about 590 nm. Measurement of the ratio of the red to green JC-1 fluorescence in cells using flow cytometry is a sensitive and specific method for monitoring changes in mitochondrial potential in living cells during induction of apoptosis by various agents. Cells were cultured in six-well plates and, after reaching approximately 70% confluence, transduced with various adenoviral vectors at various multiplicities of infection. Cells were collected using centrifugation for 5 min at 1500*g* at 4 °C and resuspended in complete medium containing 10 μg/ml JC-1 at a density of 5×10^5 cells/ml. Cells were incubated for 10 min at room temperature in the dark. Cells were then washed twice with cold PBS, resuspended in 400 μl of PBS, and analyzed immediately using flow cytometry.

Western blot analysis. Western blots were probed with a specific antibody against FHIT, p53, BID poly(ADP-ribose) polymerase (PARP), caspase 3, caspase 8, caspase 9, or β-actin. The protein bands were detected using enhanced chemiluminescence. Antibodies against p53, BID, caspase 8, caspase 9, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); FHIT polyclonal antiserum was purchased from Zymed (South San Francisco, CA); and antibodies against caspase 3 and PARP were purchased from BD Pharmingen (Los Angeles, CA).

Statistical analyses. All experiments were performed at least three times with duplicate samples. The mean values and standard errors were calculated. The StatView 5.0 (Abacus Concepts, Berkeley, CA) and SAS (SAS Institute, Cary, NC) software programs were used for all statistical analyses. Each bar denotes mean ± SEM of three experiments.

Results

FHIT overexpression targets and upregulates DR genes

We previously showed that exogenous expression of the wt-*FHIT* gene-induced apoptosis in human NSCLC cells [4,9]. To further characterize the response of NSCLC cells to exogenous expression of FHIT protein and analyze the molecular mechanism of FHIT-mediated apoptosis, we induced overexpression of wt-FHIT by transducing the human NSCLC cell lines H1299 and A549 with Ad-*FHIT*. We next used a multiprobe RPA to identify the potential upstream and downstream molecular targets of the FHIT protein in Ad-*FHIT*-transduced H1299 and A549 cells. RPA is a highly sensitive and specific method for the detection and quantitation of mRNA species. We used various sets of probes that could detect the primary factors involved in cell proliferation, apoptosis, and cell cycle regulation to quantify the transcription of genes in the apoptotic pathway that may be affected and regulated by overexpression of FHIT in Ad-*FHIT*-transduced NSCLC cells. The results showed that exogenous expression of wt-FHIT specifically targeted *DR* genes, resulting in marked upregulation of the expression of DR3, DR4, and DR5 in Ad-*FHIT*-transduced NSCLC cells (Fig. 1). We also found that the activation of DR3, DR4, and DR5 was independent of *p53* gene status in these NSCLC cells, although the effect was enhanced in the presence of p53 expression in Ad-*FHIT*-transduced A549 cells with wt-p53. The *FAS* gene was also activated by FHIT protein overexpression in A549 cells with wt-*p53* but not in H1299