

Inactivation of the *FHIT* Gene Favors Bladder Cancer Development

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ABSTRACT

The fragile histidine triad (*FHIT*) gene located on chromosome 3p14.2 is frequently deleted in human tumors. We have previously reported deletions at the *FHIT* locus in 50% of bladder carcinoma derived cell lines and reduced expression in 61% of primary transitional carcinomas of the urinary bladder. To additionally investigate the role of *FHIT* alterations in the development of bladder cancer, we used heterozygous and nullizygous *Fhit*-deficient mice in a chemically induced carcinogenesis model. Results showed that 8 of 28 (28%) and 6 of 13 (46%) of the *Fhit* $-/-$ and $+/-$, respectively, versus 2 of 25 (8%) *Fhit* $+/+$ mice developed invasive carcinoma after treatment with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. To explore the possibility of a *FHIT*-based gene therapy for bladder cancer, we studied the effects of restored *Fhit* protein expression on cell proliferation, cell kinetics, and tumorigenicity in BALB/c nude mice, with human SW780 *Fhit*-null transitional carcinoma derived cells.

In vitro transduction of SW780 *Fhit*-negative cells with adenoviral-*FHIT* inhibited cell growth, increased apoptotic cell population, and suppressed s.c. tumor growth in nude mice. These findings suggest the important role of *Fhit* in bladder cancer development and support the effort to additionally investigate a *FHIT*-based gene therapy.

INTRODUCTION

Although 60,240 bladder cancer cases will be diagnosed in the United States in 2004 (1), very little is known about its

molecular etiology. Most bladder cancers arise in the bladder epithelium and regardless of treatment with surgery, chemotherapy, or immunotherapy, may recur and/or metastasize. The search for improved therapies remains a high priority in this disease. For most experimental therapies, animal tumor models represent an essential link between *in vitro* testing and clinical trials.

Intravesical gene therapy, primarily with adenoviral-mediated gene transfer or other potential viral vectors (2), is a very promising approach for the treatment of refractory superficial bladder cancer (3). Indeed, intravesical administration enables an efficient delivery of therapeutic genes to cancer cells with minimal systemic exposure. Furthermore, the response to treatment is easily done by analysis of urine samples, cystoscopic examination, and biopsy.

Regions of homozygous and hemizygous deletion in human tumors are thought to harbor tumor suppressor genes, such as *p53* and *FEZ1*. As is the case of other solid tumors, tumor suppressor genes are thought to play a crucial role in the development and progression of bladder cancer. In 1996 we identified the *FHIT* gene in a region of the short arm of chromosome 3, at 3p14.2 (4), that is frequently deleted in bladder cancer and was likely to harbor a tumor suppressor gene (5). We then showed the importance of the *Fhit* protein as a regulator of cell growth as well as an inhibitor of tumorigenesis (6–9).

In the present study, we assessed the carcinogenic potential of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) in *FHIT* knockout mice to gain insight into the potential function of *Fhit* *in vivo*. Furthermore, to assess the potential application of *Fhit*-based gene therapy, we re-expressed *Fhit* protein in *Fhit*-negative transitional carcinoma cell lines through an adenoviral (Ad) vector and tested the effect on tumorigenicity.

MATERIALS AND METHODS

Cell Lines and Gene Transduction. Transitional cell carcinoma cell line SW780 and transformed human kidney 293 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained as recommended.

Two adenoviral vectors, an adenoviral-*FHIT-GFP* (Ad-*FHIT*) vector that encodes two separate proteins through the internal ribosome entry site, and an adenoviral-*GFP* (Ad-*GFP*) were constructed as described previously (6). Viral titers were determined by plaque assay, serially diluted infection of green fluorescent protein vector, and then observed in a confocal microscope on 293 cells and absorbance measured. SW780 TCC derived cells were transduced with standard techniques (6).

Animal Experiments. Animal experiments were conducted under institutional guidelines established for the Animal Facility at the Kimmel Cancer Center (Thomas Jefferson University, Philadelphia, PA). For the carcinogenicity study, 25 *FHIT* $+/+$ (20 males and 5 females), 13 *FHIT* $+/-$ (4 males and 9 females), and 28 *FHIT* $-/-$ mice (17 males and 11 females) of B6/129 F2 generation were treated with a freshly prepared solution of 0.1% BBN (TCI America, Portland, OR) in

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their drinking water, twice a week. The mice were weighed, observed daily, and after 13 weeks of BBN treatment followed by 2 weeks of tap water, the mice were sacrificed, and complete necropsy was conducted. At necropsy, all of the bladders were first opened and checked for gross lesions and then fixed in 10% buffered formalin. For the tumorigenicity assay, BALB/c nude mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and SW780 cells were transduced *in vitro* with Ad-*FHIT* at multiplicity of infection (MOI) of 50. Ad-*GFP* was used as a control. Transduced cells were harvested 24 hours after transduction. After determining the viability of the cell by trypan blue staining, viable cells (1×10^7) were injected s.c. into the right flank of 3 8-week-old male BALB/c nude mice in each experimental group. Mice were checked for tumor formation every other day up to 10 days. Tumor volume was determined by measuring in two directions and calculated as follows: tumor volume = length \times (width)²/2.

Histologic Analysis and Immunostaining. The whole bladder was examined. Histologic urothelial lesions were classified into four categories according to the tumor progression described in BBN induced tumors as follows: (1) simple hyperplasia and mild dysplasia; (2) moderate and severe dysplasia; (3) *in situ* carcinoma; and (4) invasive carcinoma (10, 11). The slides were randomized so that the pathologist evaluating the slides did not know the nature of the treatment received by each animal. For Fhit staining, tissue sections were deparaffinized, rehydrated in a graded alcohol series, and heated in 0.01 mol/L sodium citrate (pH 6.0) in a microwave (90°C) for 3 5-minute periods. Nonspecific binding sites were blocked with 10% normal goat serum. Sections were then incubated with rabbit anti-mouse Fhit antiserum (12) at a 1:2,000 dilution (overnight, in a humidified box), then with biotinylated goat antirabbit antiserum (1:750 dilution), and finally with streptavidin horseradish peroxidase (1:1,000 dilution). The location of Fhit protein was visualized by incubation with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO), and the sections were lightly counterstained with hematoxylin.

Immunoblot. Protein extraction and immunoblot analyses were done as described previously (13). Briefly, cells and tissues were lysed in NP40 lysis buffer, clarified by centrifugation, resolved on SDS-PAGE, and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Melville, NY). After blocking in 5% nonfat dry milk, membranes were sequentially incubated with primary and secondary antibodies. Specific signals were revealed with a chemiluminescence detection system (Amersham Life Sciences, Piscataway, NJ). The primary antibodies used were as follows: anti-Fhit at 1:1,000 (Zymed, South San Francisco, CA), rabbit polyclonal anticlaved caspase-3 at 1:1,000, mouse monoclonal anticlaved caspase-8 at 1:1,000, rabbit polyclonal anticlaved caspase-9 at 1:1,000, rabbit polyclonal anticlaved-poly(ADP-ribose) polymerase (PARP) at 1:1,000 (Cell Signaling Technology, Inc., Beverly, MA), and antiactin at 1:3,000 (Sigma, St. Louis, MO).

Flow Cytometry and Cell Counting. Flow cytometry analysis was done as described previously (13). Briefly, 5×10^5 cells were fixed in 3% paraformaldehyde, washed in PBS and stained in propidium iodide, 5 μ g/mL in PBS supplemented with RNase A (Roche, Indianapolis, IN) for 30 minutes at room temperature. Data were collected with a Coulter counter (Becton

Dickinson, Bedford, MA) and analyzed with the XL II System computer program (Becton Dickinson). For growth curve experiments, 2.5×10^5 cells were plated in triplicate in six-well plates and counted every 12 hours for 3 days. Dead cells were excluded by trypan blue staining.

Statistical Analysis. For the animal experiments, the association between *FHIT* expression and tumor formation was evaluated via the two-sided Fisher's exact test (α of 0.05). These analyses were carried out in StatXact 6 (Cytel Software Corp., Cambridge, MA, 1989 to 2003).

RESULTS AND DISCUSSION

Fhit-Deficient Mice Are More Susceptible to BBN.

Here, we have additionally investigated the role of *Fhit* alterations in the development of bladder cancer, using heterozygous and nullizygous *Fhit*-deficient mice in a chemically induced carcinogenesis model. Because the sensitivity to tumor induction characteristically depends on the target organ and the carcinogen used, we chose to use the mouse urinary bladder carcinomas induced by BBN. BBN is a potent chemical carcinogen that induces bladder tumors in mice with a yield of >90% and one of the very few chemical carcinogens showing specific carcinogenicity for the mouse urothelium (14, 15). Tumors induced by BBN are basically of the transitional cell type with a sequential progression of morphologic alterations from simple hyperplasia, to dysplasia, to carcinoma *in situ*, and finally to invasive carcinoma (10, 11). In the *Fhit* *+/+* group, simple hyperplasia and mild dysplasia were detected in 19 mice (76%), four mice (16%) showed moderate to severe dysplasia, and only two mice (8%) presented neoplastic lesions, one with foci of microinfiltration and the other with invasive carcinoma. Conversely, 6 of 13 (46%) *Fhit* *+/-* and 8 of 28 (28%) *Fhit* *-/-* mice harbored invasive carcinomas (Table 1). In most cases, we observed squamous differentiation. Mild to moderate dysplasia was diagnosed in the remaining *Fhit*-deficient mice (representative examples of gross and microscopic lesions in *Fhit*-deficient mice are shown in Fig. 1). When we used three categories of the outcome (normal, dysplasia, and carcinoma), the global *P* comparing all three groups was 0.010. In other words, we observed a different distribution of outcomes across the three groups. The pairwise *P*s were as follows: wild-type *versus* heterozygous *+/-*, *P* = 0.003; wild-type *versus* homozygous *-/-*, *P* = 0.024; and heterozygous *versus* homozygous, *P* = 0.495. Alternatively, if we analyze the outcomes as carcinoma *versus* others (*i.e.*, combine normal and dysplasia), the three groups were still significantly different from each other (\sim 8% in the wild-type group *versus* 46% in the *+/-* group *versus* 29% in the *-/-* group; global *P* = 0.023). Pairwise *P*s for this dichotomous cancer endpoint are as follows: wild-type *versus* the heterozygous *+/-*, *P* = 0.011; wild-type *versus* homozygous *-/-*, *P* = 0.081; and heterozygous *versus* homozygous, *P* = 0.307. These results showed that both *Fhit* *+/-* and *Fhit* *-/-* transitional epithelial cells are more susceptible to malignant transformation. Thus, *Fhit* appears to serve a complex role in bladder carcinogenesis, suggesting that the difference in *Fhit* function in *Fhit* *+/-* *versus* *Fhit* *-/-* bladder cells may be because of the role of *Fhit* in modulating thus far unknown partners. In fact, both the *+/-* and *-/-* groups seem to have

Table 1 Summary of the urothelial lesions observed in the three different genotypes

Genotype	Sex (m/f)	Normal/hyperplasia/ mild dysplasia	Moderate dysplasia/ severe dysplasia/ <i>in situ</i>	Carcinoma
FHIT +/+ 25	20/5	19 2 normal 9 hyperplasia 8 moderate dysplasia	4 3 moderate dysplasia 1 severe dysplasia	2 1 micro 1 deeply invasive
FHIT +/- 13	4/9	3 2 normal 1 hyperplasia	4 1 moderate dysplasia 3 severe dysplasia	6 2 micro 4 deeply invasive
FHIT -/- 28	17/11	11 2 normal 3 hyperplasia 6 moderate dysplasia	9 5 moderate dysplasia 4 severe dysplasia	8 3 micro 5 deeply invasive

elevated risk of adverse outcomes (31% dysplasias plus 46% cancers in the heterozygous group, and 32% dysplasias plus 29% cancers in the homozygous group) compared with the wild-type mice (16% dysplasias plus 8% cancers), but they are not significantly different from each other. There is some vague suggestion that cancer may be more common in the heterozygous group, but the number of animals is far too small for any such formal conclusion. The minimal difference in the incidence of bladder tumors between the two groups of mice (+/- versus -/-) raised the question of whether the wild-type *Fhit* allele was lost or epigenetically suppressed or whether *Fhit* might be haploinsufficient for tumor suppression. To determine whether

Fhit expression was silenced in tumors, we determined *Fhit* protein expression by immunohistochemistry with a rabbit anti-mouse *Fhit* antiserum (12). Of the same set of 6 tumors from *Fhit* hemizygous mice, *Fhit* staining was evident in all of the tumors (a representative example is shown in Fig. 1, right panel).

In previous studies of *N*-nitrosomethylbenzylamine-induced carcinogenesis in *Fhit* +/- mice (16, 17), immunohistochemical analysis for *Fhit* expression showed loss of the protein in *N*-nitrosomethylbenzylamine derived tumors in some cases. We concluded that the second *Fhit* allele was probably inactivated in these tumors. This would suggest that *Fhit*, like

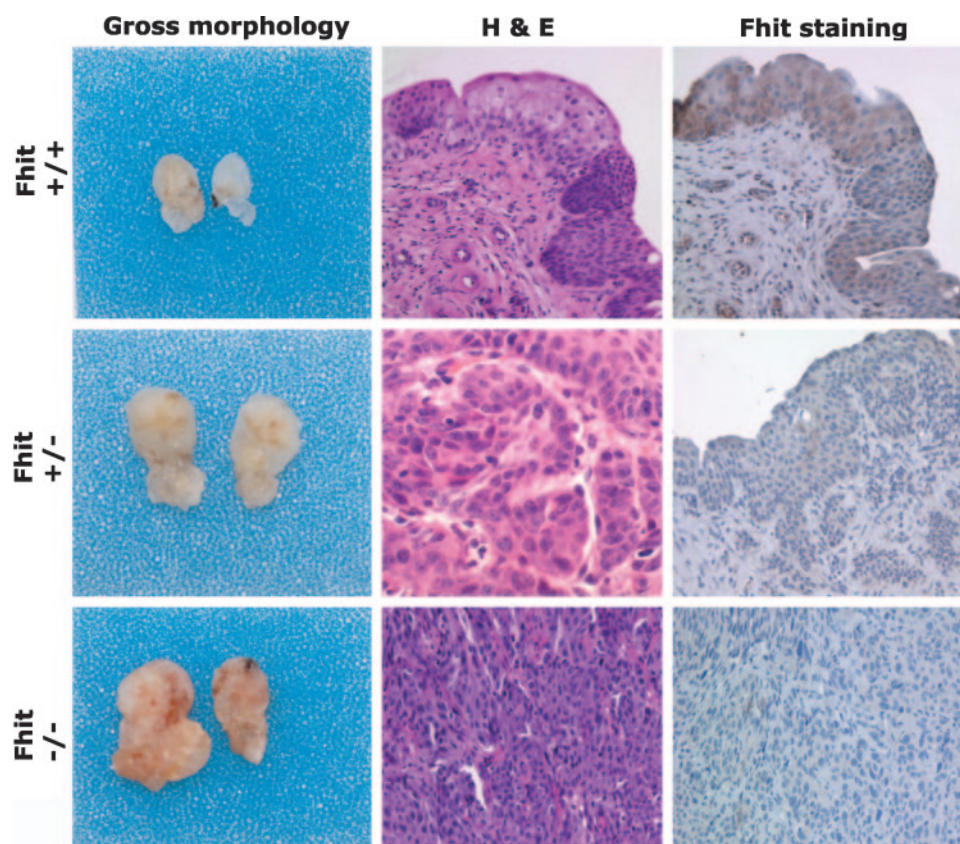


Fig. 1 Gross anatomy and histopathology of murine bladder after BBN administration. Typical features of BBN-induced pathology in *Fhit* +/+, +/-, and -/- mice (top to bottom). Gross morphology of the open bladder is shown (magnification $\times 35$; left panels). H&E stained bladder sections: normal epithelium is shown in *Fhit* +/+ mice (magnification $\times 400$), whereas *Fhit* +/- and -/- mice showed infiltrating carcinoma with foci of squamous differentiation (magnification $\times 400$; middle panels). Tumors from mice were stained for detection of expression *Fhit* protein to assess the status of *Fhit* alleles (left panels).

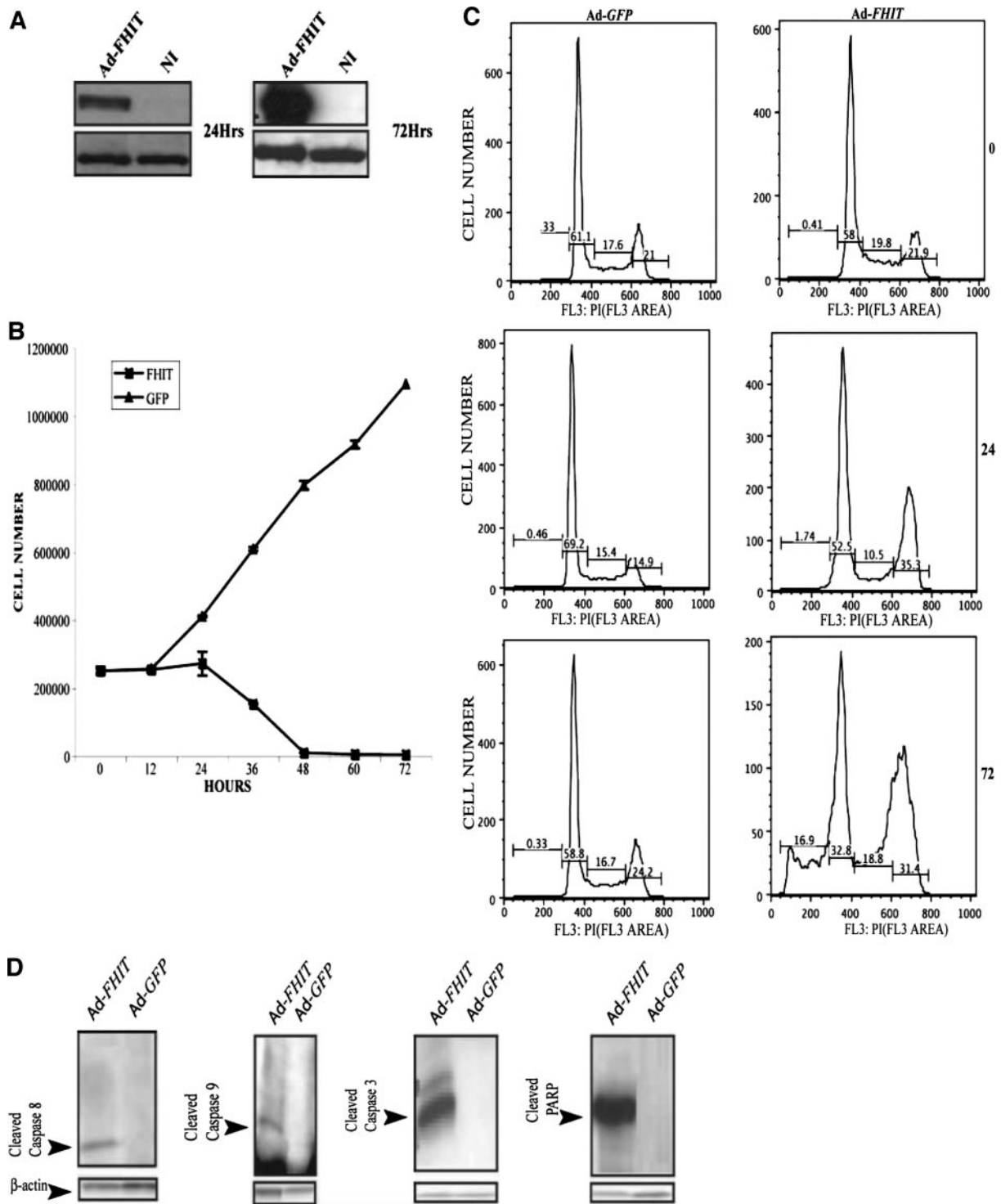


Fig. 2 Effect of Fhit reintroduction in SW780 TCC cell lines. **A**, Expression was analyzed in cell lysates extracted at 24 hours and 72 hours, after infection at MOI 50. Immunoblot analysis was done with anti-Fhit (top) and with antiactin antibody (bottom). SW780 cell line was infected with adenoviral vector for 12 hours, 24 hours, 48 hours, 60 hours, and 72 hours. Twenty-four and 72-hour Ad-FHIT-transduced cells are shown. NI (not infected) represents the same cell line noninfected. **B**, *in vitro* cell growth of TCC cancer cell line SW780. The cell numbers were counted by trypan blue exclusion at the indicated times, after transduction with Ad-FHIT, and Ad-GFP vectors at MOI 50. **C**, Flow cytometry analysis of TCC cancer cell line SW780 infected with Ad-GFP or Ad-FHIT at 0 hours, 24 hours, and 72 hours. The percentage of cell cycle distribution is described in the text. **D**, modulation of apoptotic-related protein by Ad-FHIT. Western blot shows that caspase-8 was cleaved to the active M_r 18,000 fragment. Caspase-9 was activated and cleaved in the active M_r 35,000 fragment in Ad-FHIT-infected cells. Also PARP, activated downstream by caspase-3, was cleaved. In contrast, these proapoptotic proteins were not activated in Ad-GFP-infected cells.

p27^{Kip1}, could be a haploinsufficient tumor suppressor in some tissues (17), explaining a number of early observations showing that some tumors retain an intact copy of *FHIT* and express reduced levels of Fhit protein (18).

Adenoviral Fhit Expression Affects Cell Cycle and Cell Growth in Transitional Cell Carcinoma (TCC) Cells *In vitro*.

We have previously assessed that the SW780 cell line lacks Fhit expression (19). To investigate the effects of Fhit replacement in TCC, Fhit null SW780 cancer cells were infected with an adenoviral vector expressing both Gfp and Fhit proteins (Ad-GFP-FHIT). As a control for viral infection, the same cells were infected with the same adenovirus carrying only *GFP* cDNA (Ad-GFP). Immunoblot analysis showed that 24 hours after treatment, Ad-FHIT and Ad-GFP infections resulted in substantial expression of the transgene in SW780 cells (Fig. 2A). The functional consequences of this overexpression were defined by plating SW780 cells on six-well plates and infecting them 24 hours later one time with a MOI 50 of Ad-FHIT or Ad-GFP. The cells were then counted every 12 hours up to a total of 72 hours. Growth of SW780 cells transduced with Ad-FHIT was inhibited starting from 24 hours after treatment, when compared with the growth of cells infected with Ad-GFP vector (Fig. 2B). Flow cytometry analysis indicated that the cell cycle distribution of SW780 cells was significantly affected by Fhit overexpression at 24 hours of transduction, compared with the Ad-GFP transduced controls (Fig. 2C). Indeed, the cell cycle profile indicates that 35.3% of the SW780 cells transduced with Ad-FHIT were arrested at the G₂-M phase at 24 hours post-transduction (Fig. 2C), whereas only 14.9% of Ad-GFP transduced cells were arrested at the G₂-M phase. Interestingly, at 72 hours post-transduction, 31.4% of the SW780 cells infected with Ad-FHIT were arrested at G₂-M phase with 16.9% of cells in apoptosis, whereas no apoptotic effect was observed in the Ad-GFP transduced cells (Fig. 2C). Similar results were observed in some cancer cell lines, such as TE 12 esophageal cancer cell line, H460 lung cancer cell line, and MDA-MB-436 breast carcinoma cell lines, in which we observed a marked increase of the G₂ population after Fhit reintroduction followed by a massive cell death (6, 7, 20). The precise molecular events underlying this process remain to be elucidated. Interestingly, several cell lines in which Fhit was reintroduced did not show this phenomenon (6–8), suggesting either that different pathways are involved in Fhit dependent apoptosis, maybe depending on the genetic asset, or that the threshold of Fhit expression necessary for biological effect may differ in individual cell types.

Analysis of FHIT-Induced Cell Death. We additionally investigated Fhit-dependent apoptotic activity by assessing the expression of caspase molecules, the major mediators of the classical apoptotic cascade. Specifically, we measured the modulation of activity of specific mitochondrial and cytoplasmic caspase-related proteins such as caspase-8, caspase-3, caspase-9, and PARP. To exclude virus-specific effects, we compared cell lysates from cells infected with Ad-GFP and Ad-FHIT at the same MOI. As shown in Fig. 2D, a distinct activation pattern can be discerned by comparison of the Ad-FHIT and Ad-GFP-infected SW780 cells. Initiators caspase-8 and caspase-9 were activated in Ad-FHIT-transduced cells. Western blot analysis showed that caspase-8 was cleaved to the active M_r 18,000 fragment.

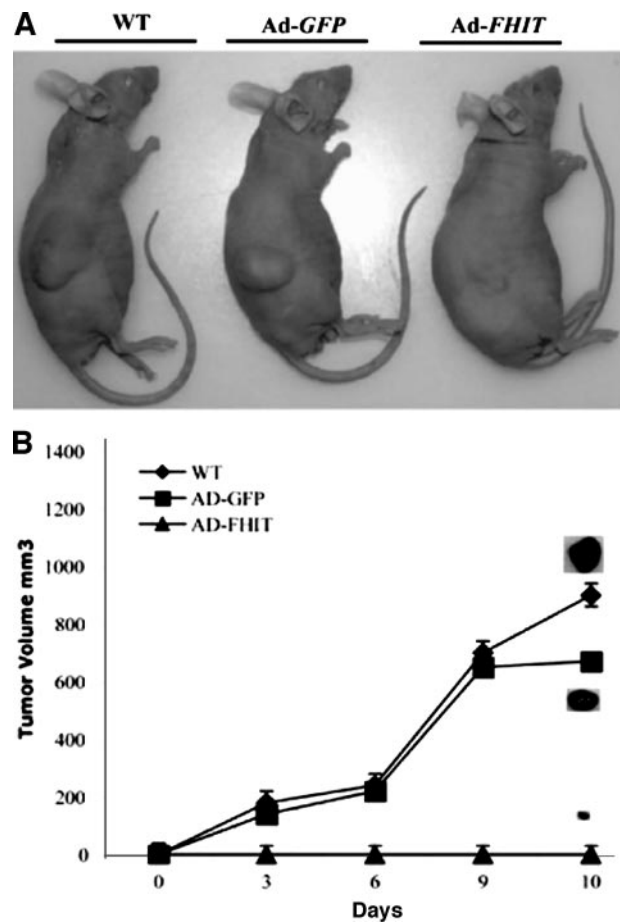


Fig. 3 Effects of Ad-FHIT-transduced SW780 on the tumorigenicity in nude mice. Tumor formation in nude mice (A) and *in vivo* growth curves (B) are shown. SW780 cells were infected by Ad-FHIT, and Ad-GFP, and injected s.c. into nude mice as described in the text. As controls, we used wild-type (uninfected) cells. (WT, wild-type)

Caspase-9, which is activated after release of cytochrome *c* from mitochondria, was activated and cleaved in the active M_r 35,000 fragment, and caspase-3 was cleaved in the active M_r 19,000/17,000 in Ad-FHIT-infected cells. The cellular protein PARP, inactivated downstream by caspase-3, was also cleaved. In contrast, these proapoptotic proteins were not activated in Ad-GFP-infected cells. Impaired apoptosis is common in cancer cells allowing mutated cells to continue to enter the cell cycle, thereby perpetuating the cycle of mutation and oncogenesis. Our results, in line with others (6–8, 20), indicate that reintroduction of Fhit with activation of the extrinsic caspase pathway may restore the proapoptotic potential of some cancer cells.

Inhibition of Tumorigenicity in Ad-FHIT-Transduced Cancer Cells. We also tested the tumorigenic potential of Ad-FHIT-transduced SW780 cells in nude mice to determine the inhibitory effects of Fhit on tumor cell proliferation *in vitro*. Viable SW780 cells (1×10^7) transduced *in vitro* at MOI 50 with Ad-FHIT or Ad-GFP for 24 hours were injected s.c. into three mice in each treatment group. Tumors were observed in all of the nontreated mice (wild-type) and in the mice bearing

SW780-Ad-*GFP*-transduced cells. Conversely, only one of three mice carrying Ad-*FHIT*-transduced cells developed a tumor (Fig. 3A). Three days post inoculation, the tumors started growing rapidly, reaching an average volume of about 900 mm³ in wild-type mice, 680 mm³ in mice who received Ad-*GFP*-transduced cells, and 200 mm³ in mice who received Ad-*FHIT*-transduced cells (Fig. 3B). The degree of prevention of tumor formation and/or growth suppression was similar to prior studies in which Ad-*FHIT* vectors induced reduction of tumor growth in esophageal, pancreatic, lung, and breast cancer (6–8, 19).

The enhanced susceptibility of *Fhit*-deficient mice to specific chemical induced bladder carcinogenesis, the *in vitro* proapoptotic effect, and the *in vivo* tumor suppressor effect exerted by *Fhit* deserve future exploration to determine its role as a possible interventional target in this malignancy.

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