Short Communication

Loss of *FHIT* Expression in Transitional Cell Carcinoma of the Urinary Bladder

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**Cytogenetic and loss of heterozygosity (LOH) studies demonstrated chromosome 3p deletions in transitional cell carcinoma (TCC). We recently cloned the tumor suppressor gene *FHIT* (fragile histidine triad) at 3p14.2, one of the most frequently deleted chromosomal regions in TCC of the bladder, and showed that it is the target of environmental carcinogens. Abnormalities at the *FHIT* locus have been found in tumors of the lung, breast, cervix, head and neck, stomach, pancreas, and clear cell carcinoma of the kidney. We examined six TCC derived cell lines (SW780, T24, Hs228T, CRL7930, CRL7833, and HTB9) and 30 primary TCC of the bladder for the integrity of the *FHIT* transcript, using reverse transcriptase–polymerase chain reaction (RT-PCR) to investigate a potential role of the *FHIT* gene in TCC of the bladder. In addition, we tested expression of the Fhit protein in the six TCC-derived cell lines by Western blot analysis and in 85 specimens of primary TCCs by immunohistochemistry. Three of the six cell lines (50%) did not show the wild-type *FHIT* transcript, and Fhit protein was not detected in four of the six cell lines (67%) tested. Fhit expression also was correlated with pathological and clinical status. A significant correlation was observed between reduced Fhit expression and advanced stage of the tumors. Overall, 26 of 30 (87%) primary TCCs showed abnormal transcripts. Fhit protein was absent or greatly reduced in 61% of the TCCs analyzed by immunohistochemistry. These results suggested that loss of Fhit expression may be as im-portant in the development of bladder cancer as it is for other neoplasms caused by environmental carcinogens.   *(Am J Pathol 2000, 156:419–424)*

Bladder cancer is the fourth most frequent cause of cancer death in men in the United States.[1] Transitional cell carcinoma (TCC) comprises 90% of all bladder cancers and is the second most frequent malignancy of the genitourinary tract. In 1999 an estimated 54,200 patients were diagnosed with bladder cancer.[2] TCC is classified histopathologically into three types: superficial, confined to the bladder wall, and invasive according to the TNM guideline.[3] Neoplasms of the transitional epithelium represent one of the first tumors to have been associated with environmental risk factors. The single largest etiological factor in the development of bladder cancer in the United States is smoking, although industrial exposure to arylamines also has been implicated as a frequent cause.[4] Numerous recent cytogenetic and molecular genetic investigations have demonstrated many genetic alterations in solid tumors, most of which affect tumor suppressor genes. The *RB, TP53, waf1/cip1,* and *CDKN2* genes have been shown to be mutated and/or deleted in a fraction of the TCC tumors of the bladder.[5–10]

Deletions of the short arm of chromosome 3 (3p) have been detected by loss of heterozygosity (LOH) and cytogenetic studies in TCC of the bladder.[11–13] Three discrete regions spanning 3p12–14, 3p21–24, and 3p24–26 often have been shown to be deleted, suggesting that these regions might harbor genes that are important in the development of TCC.[13] Vieten et al[12] have developed an *in vitro* multistep model of human uroepithelial transformation. In a study of chromosome 3 losses associated specifically with immortalization of five independent human papillomavirus-transformed urothelial cell lines, the smallest common region of deletion was 3p14.1–14.2. These results suggest that loss of a gene(s) in this region

A

B

Figure 1. The *FHIT* gene. **A:** *FHIT* exons 1–10, STSs markers analyzed by PCR (1–10), and the five BACs covering the central portion of the *FHIT* gene. **B:** Homozygous deletions of the *FHIT* gene observed in SW780, CRL7930, and CRL7833 cells.

may be responsible for immortalization of the uroepithelial cells.

We cloned the tumor suppressor gene *FHIT* at 3p14.2,[14] one of the most frequently deleted chromosomal regions in TCC of the bladder.[11] *FHIT* gene lies on a region of over 1 Mb of chromosome 3p14.2 that encompasses the *FRA3B* fragile region and encodes a protein of 147 amino acids (16.8 kd) with diadenosine triphosphate hydrolase activity.[15] A papillomavirus insertion site,[16] plasmid integration sites,[17] and cancer-specific translocation[18,19] have been mapped within the *FRA3B* fragile site in the *FHIT* gene.

The purpose of this report is to show that loss of expression of the Fhit protein occurs in the majority of transitional cell carcinomas of the bladder, suggesting that loss of *FHIT* function plays a critical role in bladder carcinogenesis.

Materials and Methods

Cell Lines and Tissues

TCC-derived cell lines (SW780, T24, Hs228T, CRL7930, CRL7833, HBT9) and 293 human kidney cells were obtained from the American Type Culture Collection (ATCC) and were maintained in the recommended media. A total of 30 transurethrally resected transitional carcinomas of the bladder were obtained from the Department of Urology, Jefferson Medical College of Thomas Jefferson University (Philadelphia, PA). Samples were taken immediately after resection and snap frozen in liquid nitrogen. In addition, sections from 85 formalin-fixed, paraffin-embedded specimens of TCC of the bladder were obtained from archival blocks of radical cystectomy cases. Forty-one of these specimens came from the Department of Urology, University of Padova (Padua, Italy), and 44 specimens came from the Department of Urology, Jefferson Medical College of Thomas Jefferson University. All samples were obtained from patients who gave informed consent to use excess pathological specimens for research purposes.

RNA Extraction and cDNA Synthesis

RNA was extracted from six TCC-derived cell lines (SW780, T24, Hs228T, CRL7930, CRL7833, HBT9) and from 30 frozen tissue samples with an RNeasy mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA. Reverse transcription (RT) was carried out in a 22-μl volume with 300 units of Superscript II (Life Technologies, Gaithersburg, MD), 500 ng/μl oligo(dT), and 50 ng/μl random hexamers. The reaction was incubated at 42°C for 50 minutes and then was boiled for 5 minutes. The final reaction was diluted with distilled water to 30 μl, and 1 μl of cDNA was used for PCR in consecutive rounds of amplification with *FHIT*-specific nested primer pairs 5U2/3D2 and 5U1/3D1, as described previously.[14]

Amplified DNA fragments corresponding with full-sized and aberrant *FHIT* transcripts in the cell lines were excised from agarose gel, purified, and sequenced directly. Sequence analysis was performed on 373A and 377 DNA sequencers (Applied Biosystems, Foster City, CA.)

DNA Analysis

DNA from six TCC-derived cell lines (SW780, T24, Hs228T, CRL7930, CRL7833, HBT9) was extracted by standard techniques and amplified by PCR, using oligonucleotide primer pairs for 10 loci within introns 3 (12f/r for 1; 09f/r for 2), 4 (06f/r for 3; 03f/r for 4; Col10kf/r for 5; 0817EF/ER for 6), and 5 (36C3/36C4 for 7; N143kf/r for 8; N100Kf/r for 9; N020Kf/r for 10) and for exons 5, 7, 8, and 9 of the *FHIT* gene (Figure 1). PCR amplification reactions were performed in a 25-μl volume, using 100 μg of tem-

plate, 1 unit of *Taq* polymerase (Takara, Pan Vera Corp., Madison, WI), 20 ng of primers, and 0.5 mmol/L of each deoxynucleoside triphosphate. Amplifications were carried out in a Perkin-Elmer thermal cycler for 35 cycles of 94°C for 20 seconds (for denaturation), 57°C for 30 seconds (varied for specific primer pairs), and 72°C for 30 seconds (for extension).

Amplified products were resolved on a 1.5% agarose gel.

Protein Extraction and Immunoblot Analysis

Cell lines from which protein was extracted were pelleted and rinsed with phosphate-buffered saline (PBS) (Gibco, Grand Island, NY) and were lysed in 400 μl of protein lysis buffer (10 mmol/L Tris, 1% NP-40, 0.1% bovine serum albumin, 1 mmol/L EDTA, PBS) and 50 μl phenyl-methylsulfonyl fluoride (PMSF) (125 mmol/L PMSF in ethanol) on ice for 5 minutes. After a 6-minute centrifugation at 8500 rpm at room temperature, the lysate supernatant was removed and was assayed for protein concentration with the Protein Assay reagent (Bio-Rad Laboratories, Melville, NY). Protein was aliquoted and stored at −80°C.

For Western analysis, 100 μg of protein was boiled in loading buffer containing 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), glycerol, bromophenol blue, and β-mercaptoethanol for denaturing. Samples were electrophoresed on 4–20% precast Tris-glycine gels (Fisher Scientific, Pittsburgh, PA) with the Mini Protean II electrophoresis system (Bio-Rad Laboratories) and subsequently transferred to a nitrocellulose membrane (MSI, Westborough, MA) with the Mini *trans*-Blot cell apparatus (Bio-Rad Laboratories). Membranes were blocked for 3 hours at room temperature in 5% nonfat dry milk dissolved in TBS-T (Tris-buffered saline Tween20). Primary and secondary antibodies were diluted in 5% nonfat dry milk solution. Western analysis was performed overnight at 4°C with a 1:1000 dilution of the Fhit polyclonal antibody (Zymed Laboratories, San Francisco, CA). Secondary antibody solution (1:2000 dilution) containing anti-rabbit immunoglobulin labeled with horseradish peroxidase (Amersham Life Sciences, Buckinghamshire, England) was applied for 1 hour at room temperature. Membranes were treated with enhanced chemiluminescence detection reagents (Amersham Life Sciences) according to the manufacturer's recommended protocol. Bands were detected by exposing the membrane to medical X-ray film (Fuji, Stamford, CT) for 1–10 minutes at room temperature.

Blots were stripped in a solution containing 100 mmol/L β-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7) for 30 minutes at 60°C. They were rinsed in TBS-T and reprobed for protein standardization under the previously mentioned conditions, with β-actin (Sigma, St. Louis, MO) as the primary antibody and a 1:2000 dilution of the anti-mouse secondary antibody labeled with horseradish peroxidase (Amersham Life Sciences), and exposed for 1 minute at room temperature before the film was developed.

Immunohistochemistry

Routine deparaffinization of all sections mounted on positive charge slides was carried out according to standard procedures, followed by rehydration through an ethanol series. The slides were immersed in citrate buffer (0.01 mol/L sodium citrate, pH 6.0) and heated in a microwave oven at 600 W (three times for 5 minutes) to enhance antigen retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase in methanol for 30 minutes. The sections were then incubated with anti-Fhit antibody, 1:1000 dilution (Zymed Laboratories) overnight at room temperature. The primary antibody was omitted and replaced by PBS in the negative control. After this incubation, sections were treated with biotinylated anti-rabbit antibody and streptavidin-biotin-peroxidase (Histostain-SP Kit; Zymed Laboratories). Antibody localization was detected, with diaminobenzidine as a chromogen substrate. Finally, sections were washed in distilled water and weakly counterstained with Harry's modified hematoxylin.[20]

Statistical Analysis

We used the Fisher exact test with a two-tailed *P* for analysis of statistical significance of correlation between clinicopathological parameters and Fhit expression.

Results

### Homozygous Deletions in TCC-Derived Cell Lines

To determine whether genomic deletions occur in TCC cell lines, we analyzed six TCC-derived cell lines (SW780, T24, Hs228T, HBT9, CRL7833, CRL7930) by PCR amplification, using oligonucleotide primers appropriate for 10 STSs placed in introns 3, 4, and 5 and for exons 5, 7, 8, and 9 of the *FHIT* gene.

Three cell lines (SW780, CRL7930, CRL7833) exhibit discontinuous homozygous deletions. As shown in Figure 1, the fragments that are commonly deleted overlap with regions of the *FHIT* gene that were deleted in previously reported cancer-derived cell lines.[14,19] Homozygous deletions did not include *FHIT* exons 5, 7, 8, and 9.

The three cell lines with homozygous deletions within the *FHIT* gene are the same three cell lines showing both absent or abnormal *FHIT* transcript and an absence of Fhit protein (see below).

Expression of FHIT Transcripts in TCCs

Using the RT-PCR amplification assay, *FHIT* cDNAs of altered size were identified in one (CRL7833) of the six cell lines (data not shown). Four cell lines (SW780, T24, Hs228T, HBT9) showed the presence of only a normal-sized *FHIT* transcript. *FHIT* cDNA could not be amplified from CRL7930 cells. Sequencing of the amplified DNA products revealed normal *FHIT* sequences in T24,
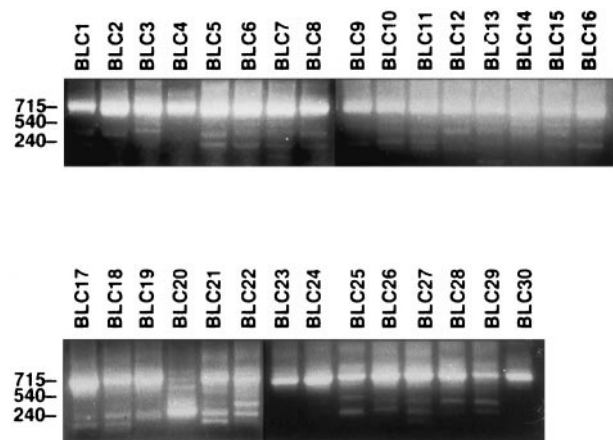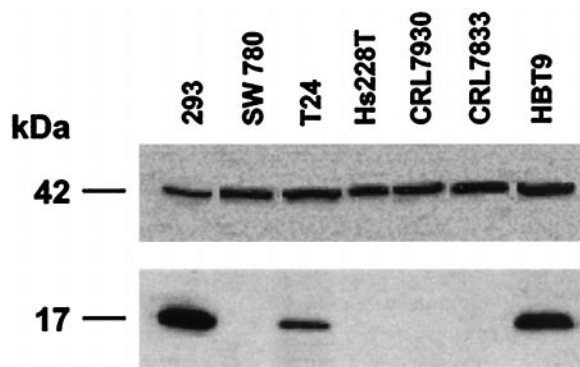
Figure 2. Analysis of expression of the *FHIT* gene by nested RT-PCR. *FHIT* RT-PCR products in 30 primary TCCs are shown. Case names are shown at the **top**; the size of the amplified product is to the **left**. The 715-bp band represents the size of the wild-type *FHIT* transcript.

HS228T, and HBT9 cells. The normal-sized band present in SW780 consisted of a mixture of abnormal *FHIT* transcripts.

Similar experiments then were performed using RNA extracted from primary TCCs of the bladder. Overall, 26 of 30 (87%) primary TCCs showed a combination of normal and one or more aberrant transcripts (Figure 2). Abnormalities of the *FHIT* transcripts consisted of the absence of one or more exons of the *FHIT* gene or the absence of exons with insertion of intronic sequences, as described previously.[14,19]

Expression of Fhit Protein in TCCs

Immunoblot experiments using protein lysate from the six transitional carcinoma-derived cell lines showed an absence of Fhit protein in the three cell lines (SW780, CRL7930, CRL7833) with absent or abnormal *FHIT* transcripts (Figure 3). Conversely, two cell lines, T24 and HBT9, expressed abundant Fhit protein in concordance with the apparent absence of *FHIT* gene alteration in these cells. The absence of Fhit protein in Hs228T cells in

Figure 3. Expression of the Fhit protein in TCC cell lines. Western blot analysis of protein from TCC-derived cell lines with anti-Fhit antiserum is shown. The size of the protein is shown at the **left**. **Bottom:** Absence of the Fhit protein in SW780, Hs228T, CRL7930, and HBT29 protein lysates; the 293 lysate is from a transformed human kidney cell line previously shown to express Fhit.[18] **Top:** β-Actin expression for the same membranes.

Table 1. Clinical-Pathological Features and Fhit Expression

Case	Sex	T		G	Fhit
26 (30.6%)	M 23 F 3	4TA 5T3b 7T2 IT3/4 1T2a 3T4a 5T3		1 Cis 2G2/3 2 G1 15 G3 1 G1/2 5 G2	−
17 (20%)	M 14 F 3	1TA 5T3b 6T2 2T4a 3T3		1 G1 3 G2 13 G3	−/+
9 (10.6%)	M 5 F 4	5TA 3T1 1T2		1 Cis 2 G1 1 G1/2 3 G2 2 G2/3	+/−
33 (38.8%)	M 26 F 7	10TA 1T3b 7T1 6T4a 9T2		1 Cis 2 G1 4 G1/2 11 G2 1 G2/3 13 G3 1 G4	+

discordance with the presence of a normal *FHIT* transcript might be because of the expression of a low level of *FHIT* transcript, as described previously for RCC cell lines.[20]

Primary TCCs were tested for expression of Fhit protein by immunohistochemical analysis with the Fhit-specific polyclonal antiserum. The 85 TCC specimens listed in Table 1 were characterized for tumor grade and stage, and sections were assessed for Fhit expression.

Sections containing a portion of normal urothelium, representing an internal positive control, were analyzed (Figure 4). A four-tier scoring system was used: 100% Fhit-positive cells were scored as +, >50% positive as +/−, <50% positive as −/+, and 98–100% negative as −.
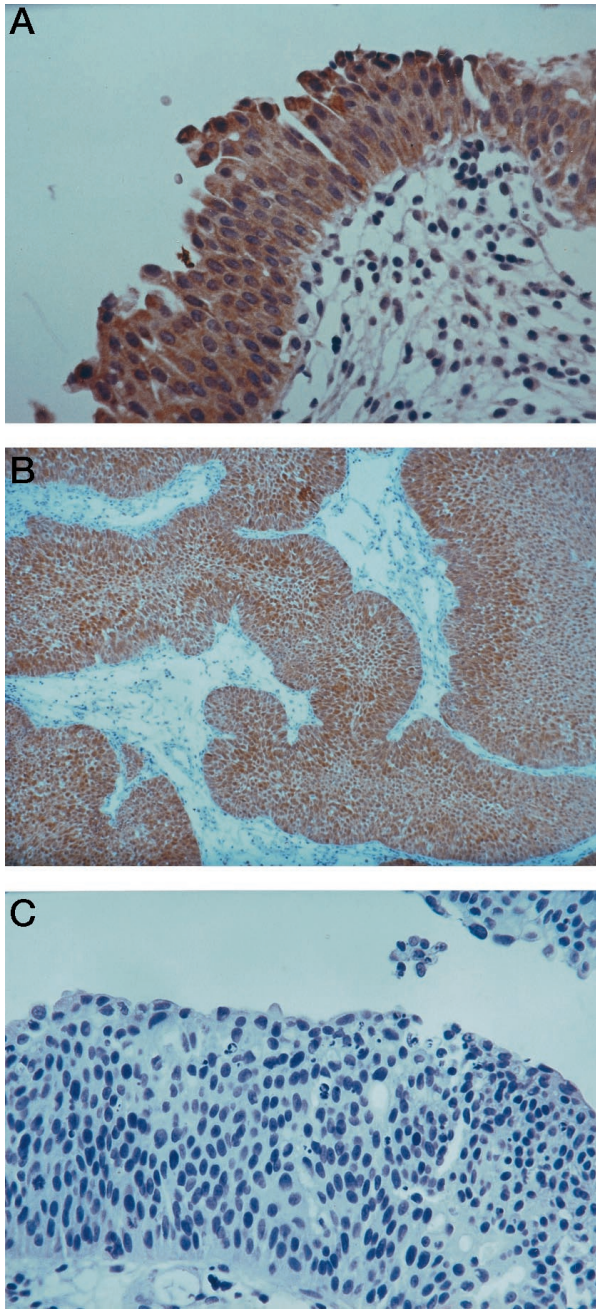
Examples of Fhit expression in normal urothelium and TCC sections are shown in Figure 4. Fhit protein was undetectable in 26 of 85 (30.6% −) specimens, 17 (20% −/+) specimens showed <50% Fhit-positive cells, and nine specimens (10.6% +/−) showed >50% Fhit-positive cells. Diffuse Fhit immunostaining in all tumor cells was observed in 33 (38.8% +) of the specimens.

Statistical Analysis

Statistical studies showed a significant correlation ($P = 0.0013$) between absence and/or reduction of Fhit expression and advanced tumor stage. No correlation was found between Fhit expression and tumor differentiation grade.

Discussion

Many studies have documented genetic aberrations in the development of human transitional cell carcinoma of the urinary bladder, including point mutations of the *HA-*

Figure 4. Fhit immunostaining in primary TCCs. **A:** Uniform strong staining of normal urothelium (×250). **B:** Papillary TCC showing Fhit expression (×120). **C:** Complete absence of Fhit staining in a superficial TCC (×250).

RAS-1 gene,[21] amplification and/or overexpression of the EGF receptor,[22] mutation and deletion of *RB*, *TP53*, *waf1/cip1,* and *CDKN2* genes,[5–10] and as yet unidentified genes on regions of chromosomes 8p and 9p. Allelic losses of chromosome 3p have been reported, and the common regions of allelic loss appear to be 3p12–14, 3p21–24, and 3p24–26.[11–13] The tumor suppressor gene(s) targeted by 3p allelic losses in transitional cell carcinoma of the bladder, however, have not been identified. Because one of the regions of 3p frequently deleted in TCCs contains the *FHIT* locus (3p14.2), our finding of reduced or absent Fhit protein expression in most

TCC-derived cell lines and in primary tumors suggests that *FHIT* is a tumor suppressor gene involved in the pathogenesis of TCC of the bladder. The *FHIT* gene also has been shown to be disrupted by translocation[14] in members of a family with heritable renal cell carcinoma that segregates with the t(3;8) (p14.2;q24) chromosome translocation.[23] One family member with the translocation died from bladder cancer and undifferentiated abdominal adenocarcinoma.[23]

Western blot analysis shows absence of Fhit protein in four of six TCC-derived cell lines examined. The observation of multiple separate homozygous deletions that did not include *FHIT* exons in three of the four cell lines showing absence of Fhit protein suggested that in these cells the homozygous deletions are the result of an overlap of independent deletions of the two *FHIT* alleles, as previously demonstrated in other tumors.[24,25] The immunohistochemical study of 85 primary TCCs of all grades and stages has demonstrated that 30.6% of them were uniformly negative and another 30.6% were a mixture of Fhit-positive and -negative cells. Thus 61.2% of these primary tumors show reduced expression of Fhit. In addition, RT-PCR analysis of the integrity of *FHIT* transcripts in primary tumors and derived cell lines suggests frequent gene lesions. Similarly, 85% of clear cell renal carcinomas and more than 70% of cervical carcinomas have shown loss or reduction of Fhit expression.[20,26] The inactivation of *FHIT* in bladder tumors is probably a result of deletions within both alleles, as described for other carcinomas, particularly tumors resulting from exposure to environmental carcinogens. These include cancers of the lung, esophagus, stomach, cervix, and pancreas.[14,26–32] Further studies will be required to define the exact mechanisms leading to loss of Fhit in bladder cancer.

Our study of transitional cell carcinoma of the bladder shows a significant correlation ($P = 0.0013$) between the absence or reduction of Fhit expression and advanced stage of the primary tumor. The heterogeneous pattern of Fhit loss of expression in TCCs of the bladder is similar to the pattern observed in breast carcinoma.[33] In addition, reduced Fhit expression is correlated with a more aggressive disease in both bladder and breast cancers. Conversely, a uniform pattern of loss of Fhit expression has been observed in tumors such as lung and gastric cancers, in which loss of Fhit has been suggested to be an early event in the tumorigenesis process.[34,35] In conclusion, we show for the first time that *FHIT* is inactivated in the majority of transitional cell carcinomas of the urinary bladder and suggest that *FHIT* inactivation is a late event in neoplastic progression.

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