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Advances in Brief

FHIT Gene Expression in Human Ovarian, Endometrial, and Cervical Cancer Cell Lines[1]

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Abstract

The *fragile histidine triad* (*FHIT*) gene, located at 3p14.2, has been shown to be altered in numerous epithelial cancers. Because previous studies have shown a loss of heterozygosity and cytogenetic abnormalities at the 3p region in ovarian, endometrial, and cervical carcinomas, we examined the status of the *FHIT* gene in 14 ovarian, 8 cervical, and 4 endometrial human cancer cell lines. RNA was isolated and subjected to reverse transcription-PCR to amplify the *FHIT* gene transcript. Sixty-three % (5 of 8) of cervical cell lines, 14% (2 of 14) of ovarian cell lines, and none (0 of 4) of the endometrial cell lines displayed aberrantly migrating FHIT transcripts. DNA sequencing demonstrated that the aberrantly migrating bands primarily lacked exons 5, 6, and 7 (with other exon losses also observed), resulting in shorter mRNA transcripts. Southern blot analysis of DNA from five of the cervical carcinomas demonstrated alterations in four of them, three of which had exhibited no normally sized FHIT transcripts. The results suggest that the expression of the *FHIT* gene may be altered in cervical tumor tissue, potentially implicating this gene in cervical tumorigenesis, whereas the involvement of this gene appears to be less important in the development of ovarian and endometrial cancer.

Introduction

Gynecological cancers remain a significant health problem for women in the United States, with 82,100 new cases and 26,900 deaths estimated for 1996 (1). Despite substantial advances in our understanding of the molecular events involved in the development of many epithelial cancers, such as colon, breast, and lung cancers, the molecular origins of gynecological cancers remain essentially unknown. This is particularly relevant for cervical cancer, which is one of the major causes of cancer death for women in many parts of the world. In addition, the absence of an effective screening tool for the early diagnosis of ovarian cancer is the main reason for the high mortality rate associated with this disease, with 14,800 deaths predicted for 1996 in the United States (1). A great deal of effort is currently aimed at understanding the basic molecular biology of these tumors, with the ultimate goal being the identification of early diagnostic markers, prognostic indicators, or therapeutic strategies that can be applied in the clinical context.

The *FHIT*[4] gene was recently described as a candidate tumor suppressor gene implicated in the development of various epithelial cancers, including esophageal, gastric, lung, head and neck, and Merkel cell cancers (2–5). Approximately 50% of these tumors display aberrant *FHIT* gene transcripts, which lack exons considered relevant for the normal functioning of the protein product. This gene is located at 3p14.2, close to a chromosomal region shown to be susceptible to LOH and cytogenetic abnormalities in ovarian, endometrial, and cervical cancers (6–8). This evidence prompted us to examine the status of the *FHIT* gene in established cell lines derived from ovarian, endometrial, and cervical tumor tissue. Evidence of *FHIT* gene abnormalities in the cell lines could suggest involvement of this gene in the development of gynecological cancer and would warrant further investigation in primary tumor tissue.

Materials and Methods

Cell Lines and Tissue. The *FHIT* gene was analyzed in a total of 14 ovarian, 8 cervical, and 4 endometrial cancer cell lines (Table 1). Endometrial and cervical cell lines were obtained from the American Type Culture Collection. The ovarian cell lines were obtained as generous gifts from Dr. Scott Rose (University of California, San Diego, CA), Dr. David Spriggs (Memorial Sloan Kettering Cancer Center, New York, NY), and Dr. Jacques De Greve (AZ-VUB-Oncologisch Centrum, Brussels, Belgium). All cell lines obtained from the American Type Culture Collection were cultured as recommended. Ovarian cell lines (except the cell lines 420 and 432) were cultured in RPMI containing 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The cell lines 420 and 432 were cultured in DMEM containing 10% (v/v) heat-inactivated calf serum, 2.5 units/liter insulin, and 50 μg/ml gentamicin (Life Technologies, Inc., Gaithersburg, MD). Normal gynecological tissue samples (two endometrial, one ovarian, and 3 cervical) were collected from three patients who were operated on for benign conditions, and blood was obtained from four healthy volunteers. All samples were obtained with informed consent.

RNA Isolation and RT. Total RNA was prepared from cultured cells and tissue samples using the standard guanidium thiocyanate CsCl approach (9). Single-strand cDNA synthesis was performed essentially as described (3, 5) with the following modification: 80 ng/μl (235 pmol) of primer 3D2 (5′-TCACTGGTTGAAGAATACAGGA-3′; Ref. 2) was used in the RT assay. Tubes containing RNA and primer were heated at 70°C for 10 min and immediately chilled on ice prior to the addition of 5× first strand buffer, DTT, dNTPs, reverse transcriptase (SuperScript II; all reagents from Life Technologies, Inc.), and RNasin (Promega, Madison, WI). Samples were incubated at 37°C for 60 min, and the reaction was stopped by incubation at 94°C for 5 min. Control reactions in which the reverse transcriptase was omitted were included with all assays.

PCR Amplification. Two different PCR assays were used to assess the status of the *FHIT* gene transcript. The first, based on the nested PCR approach reported by Ohta *et al.* (2), contained 0.8 μM primers (3D2; see above and primer 5U2, 5′-ATCCTGGAAGCTTTGAAGCTCA-3′), 50 μM each dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1.25 units of Taq polymerase (all reagents from Life Technologies, Inc.) in a final volume of 25 μl. The reaction was cycled 25 times using the conditions described (2) in a Perkin-Elmer thermal cycler (model 9600). Diluted PCR product (1 μl of a 20-fold dilution) was used in a subsequent nested PCR assay in a final volume of 25 μl using the primers 5U1 (5′-TCCGTAGTGCTATCTACATC-3′) and 3D1 (5′-CATGCTGATTCAGTTCCTCTTGG-3′; Ref. 2). The same conditions described above were used, with the exception that the primer 5U1 was used at a final concentration of 3.2 μM, and the reaction was allowed to proceed for 30 cycles. Analysis of

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[4] The abbreviations used are: FHIT, fragile histidine triad; LOH, loss of heterozygosity; RT, reverse transcription.

Table 1 *FHIT gene expression in human endometrial, cervical, and ovarian cancer cell lines*

Cell line	*FHIT* gene transcript	Exons absent/insertions
Endometrial		
Hec 1A	N[a]	
AN3-CA	N	
RL95-2	N	
KLE	N	
Cervical		
ME-180	N	
MS751	Ab	5, 6, 7, & 8 and 88-bp insertion
SiHa	Ab	5, 6, & 7 and 5, 6, 7, & 8
C-33A	Ab	5, 6, 7, & 8
C4I	Ab	4, 5, 6, & 7
HT-3	Ab	4, 5, 6, & 7
CaSki	N	
HeLa	N	
Ovarian		
NIHOvCar 3	N	
222	N	
CP70	Ab & N (reduced)	5, 6, 7, & 8
UCI 101	N	
A2780	N	
432	N	
420	N	
AD10	N	
UCI 107	Ab & N (reduced)	5 & 6 and 5, 6, 7, & 8
SKOV 3	N	
A547	N	
PA1	N	
A364	N	
A224	N	

[a] N, normally sized transcript of *FHIT* gene; Ab, FHIT transcripts consistently shorter than expected.

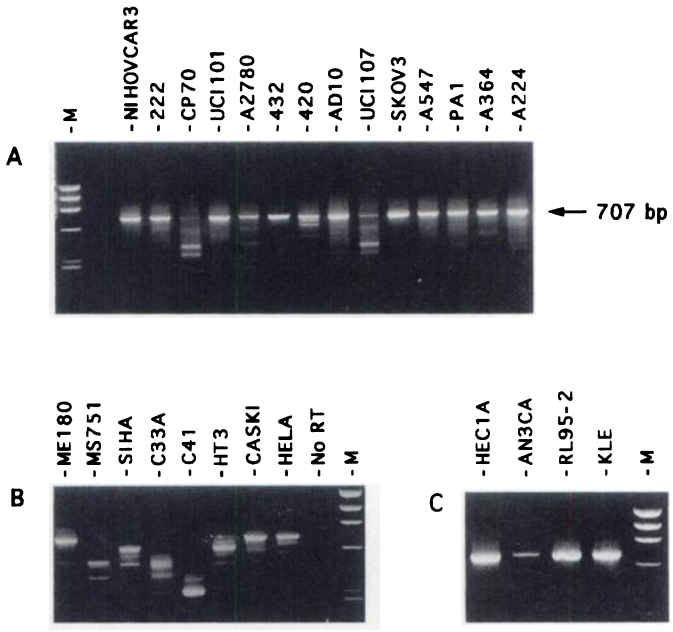

Fig. 1. RT-PCR analysis of the *FHIT* gene product in ovarian (*A*), cervical (*B*), and endometrial (*C*) cell lines using a nested PCR assay. Total RNA isolated from cell lines was subjected to RT-PCR (inner primers 5U1 and 3D1; Ref. 2), and PCR products were separated on 2% agarose gels. *Arrow*, the 707-bp *FHIT* gene product (*A*). Lanes containing molecular weight markers (ØX174 DNA digested with *Hae*III) are shown (*Lanes M*), as well as control points from which reverse transcriptase had been omitted (*Lane No RT*).

the *FHIT* gene transcript was repeated for all cell lines using another set of primers, MUR5 (5′-CTGTAAAGGTCCGTAGTGC-3′) and RP2 (5′-ACAGGATGGTGAGATGAAGAAACTGC-3′; Ref. 10). We used PCR conditions similar to those described by Thiagalingam *et al.* (10), with 60 mM Tris-HCl (pH 9.5), 15 mM $(NH_4)_2SO_4$, and 2 mM $MgCl_2$ (final concentrations). The *FHIT* gene transcript was visualized with one round of PCR amplification using the latter conditions. Primer MUR5 partially overlaps primer 5U1 in exon 3, and primer RP2 partially overlaps primer 3D2 in exon 10 (see Refs. 2 and 10).

Gel Electrophoresis and DNA Sequencing. PCR products were electrophoresed on 2% agarose gels (NuSieve 3:1 agarose; FMC Bioproducts, Rockland, ME), and DNA fragments of interest were excised. Eluted fragments were amplified by PCR in a volume of 50 μl, using the primers 5U1 and 3D1 under the same conditions described for those specific primers (see above). PCR products were separated from reactants using the QIAquick Gel Extraction Kit (Qiagen, Inc. Chatsworth, CA) and either sequenced directly using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) on an Applied Biosystems DNA sequencer (model 373A) or cloned (TA cloning kit; Invitrogen, San Diego, CA) prior to sequencing.

Southern Blotting. Genomic DNA was isolated using a standard phenol/chloroform method and treated with RNase, and 5 μg was digested with *Bam*HI (8 units/μg DNA) for 2 h at 37°C. The digested DNA was electrophoresed through 0.8% agarose, transferred to Nytran N^+ membrane, prehybridized, and hybridized according to the manufacturer's recommendations (Schleicher & Schuell, Keene, NH). The blot was probed with ^{32}P-labeled FHIT (cDNA PCR product, exons 1–9; a generous gift from Dr. K. Huebner, Kimmel Cancer Institute, Philadelphia, PA), which had been labeled using the Prime-It II Kit (Stratagene, La Jolla, CA). Hybridized blots were processed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to visualize the radioactive bands.

Results

We analyzed 26 gynecological cancer cell lines by RT-PCR for *FHIT* gene expression. All cell lines expressed *FHIT* gene transcripts. A PCR product of 707 bp (primers 5U1 and 3D1), corresponding to the expected size of the FHIT RT-PCR product (2), was observed in 73% (19 of 26) of the cell lines tested (Fig. 1). This band (707 bp) was consistently either absent or much weaker (<10%) in the remaining cell lines tested (2 of 14 ovarian and 5 of 8 cervical cell lines). The same cell lines that lacked or displayed substantially reduced levels of the normal *FHIT* gene transcript also consistently displayed aberrant *FHIT* gene transcripts [63% (5 of 8) of the cervical and 14% (2 of 14) of the ovarian cell lines]. None of the endometrial cell lines (zero of four) examined in this study displayed aberrant *FHIT* gene transcript products (Table 1 and Fig. 1). In addition, no aberrant *FHIT* gene

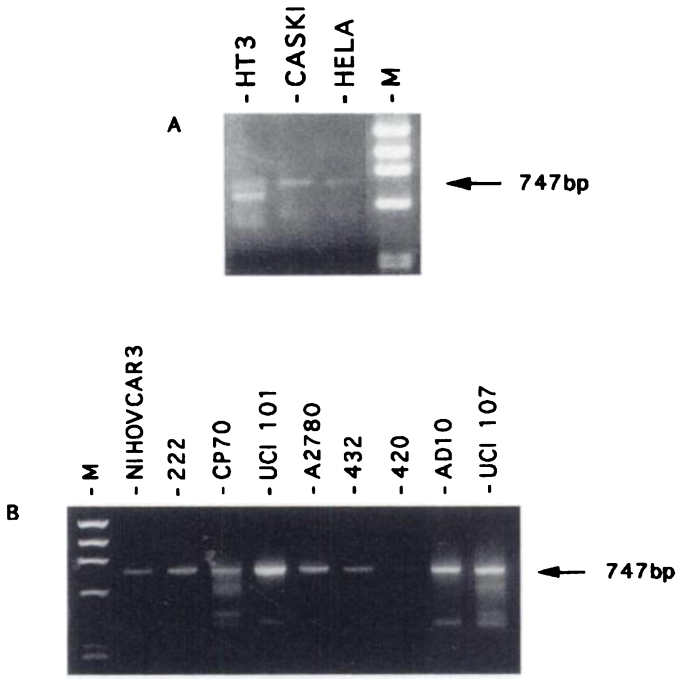

Fig. 2. RT-PCR analysis of the *FHIT* gene product in cervical (*A*) and ovarian (*B*) cell lines using the primers MUR5 and RP2 located in exons 3 and 10, respectively (10). *Arrows*, the 747-bp PCR product. *Lane M*, a molecular weight marker (ØX174 DNA digested with *Hae*III).

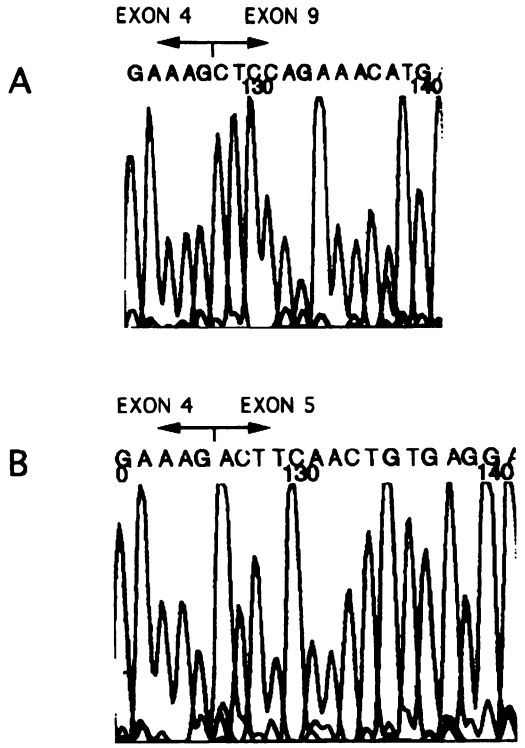

Fig. 3. *A*, partial DNA sequence of the *FHIT* gene transcript from SiHa (cervical cell line) showing the absence of exons 5, 6, 7, and 8. *B*, the expected DNA sequence for this region of the *FHIT* gene transcript.

transcripts were observed in the 10 normal tissue samples tested (two endometrial samples, three cervical samples, one ovarian sample, and four blood samples) obtained from seven individuals (data not shown). Cell lines were only scored positive for aberrant FHIT transcripts if PCR products of altered size were reproducibly observed in PCR assays from at least two separate RT reactions and confirmed using a different set of PCR primers (Fig. 2). The criteria described were conservative and could underestimate cell lines scoring positive for aberrant FHIT transcripts. For instance, the ovarian cell line 420 was defined as normal, despite the pattern shown in Figs. 1*A* and 2*B*. The second set of primers (MUR5 and RP2; Ref. 10) amplified a fragment of the FHIT cDNA that was nearly identical to, but 40 bp longer than, the fragment generated by 5U1 and 3D1 (2). Other PCR products, smaller than 707 bp, were also frequently observed together with the normally sized (707 bp) FHIT RT-PCR product (Fig. 1, *A* and *B*). However, these minor bands were not reproducibly seen and stained much less intensely than the 707-bp fragment.

DNA sequencing of the aberrant PCR products revealed FHIT transcripts that lacked the exons specified in Table 1. The two affected ovarian cell lines, CP70 and UCI 107, displayed FHIT transcripts that lacked exons 5, 6, 7, and 8. Exons 5, 6, and 7 were most commonly absent in the *FHIT* gene transcript in the affected cervical cell lines, with some cell lines also displaying lack of exons 4 and/or 8. Fig. 3 shows a typical DNA sequence obtained for an aberrant fragment (from SiHa), with exon 4 contiguous with exon 9 (Fig. 3*A*, SiHa), compared to the expected sequence for this region of the *FHIT* gene transcript (Fig. 3*B*). In addition to the absence of exons 5, 6, and 7 in the cervical cell line MS751, we also detected an 88-bp insertion between exons 4 and 8. The sequence of this 88-bp insertion differed from the 158-bp insertion between exons 4 and 5 reported for tissue from a benign breast lesion (11). We also observed polymorphic splicing of the *FHIT* gene transcript in exon 10 at the exon 9/10 border in many cell lines, as reported previously (3, 12).

Southern blot analysis (Fig. 4) of genomic DNA digested with *Bam*HI suggested that the structure of the *FHIT* gene was altered (*arrowheads*) in four of the cervical cell lines examined in this study (SiHa, MS751, C33A, and HeLa). Structural abnormalities of the *FHIT* gene were not observed in the HT3 cell line (Fig. 4), which displayed aberrant FHIT transcripts by RT-PCR analysis (Fig. 1).

Discussion

Our results show that the expression of the *FHIT* gene was altered in 63% (5 of 8) of the cervical, 14% (2 of 14) of the ovarian, and none (0 of 4) of the endometrial cancer cell lines examined. No normal *FHIT* gene transcripts were observed in the affected cervical cell lines (MS751, SiHa, C33A, C4I, and HT3), suggesting that both alleles of the *FHIT* gene were altered. The presence of *FHIT* gene abnormalities in the cell lines implies either that the abnormalities were present in the cervical tumor tissues from which the cell lines were derived or that the abnormalities reflect changes incurred during cell culture. Earlier studies using primary cervical tumor tissue report 75% LOH at 3p13–14.3 (6) and 91% LOH at 3p14 (7), suggesting that the first hypothesis may be correct. We are currently assessing the status of the *FHIT* gene transcript in paired tumor and normal cervical tissue to address this issue in cervical cancer.

If the *FHIT* gene were significantly involved in ovarian or endometrial tumorigenesis, then a signature of its involvement should persist in cell lines derived from these two tumor types. Because only 14% (2 of 14) of ovarian cell lines and none of the endometrial cell lines (0 of 4) displayed abnormalities of the *FHIT* gene transcript, we would suggest that the *FHIT* gene is minimally involved in ovarian and endometrial tumorigenesis. Previous studies showing LOH at the 3p region in endometrial and ovarian tumor tissue (6, 8) may reflect the presence of other relevant genes in this region.

We also observed minor PCR products that were shorter than the expected *FHIT* gene transcript in many cell lines. The presence and size of these bands were not reproducible in the nested PCR assay and

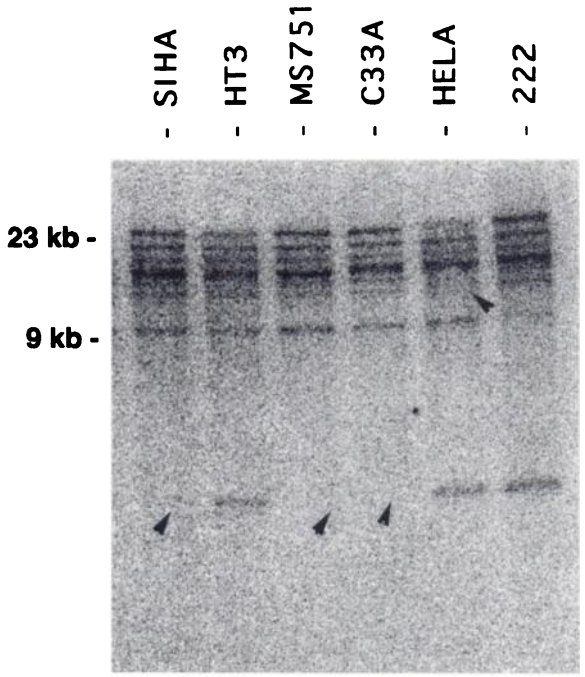

Fig. 4. Southern blot of genomic DNA isolated from cervical (*Lanes SiHa, HT3, MS751, C33A*, and *HeLa*) and ovarian (*Lane 222*) cell lines. Five μg of DNA were analyzed per lane, as described in the "Materials and Methods" section. *Arrowheads*, altered restriction fragments.

were not observed in the single-round PCR assay. The relevance and origin of these bands are unknown.

The pattern of altered FHIT expression seen in this study is similar to the abnormalities observed in digestive tract cancers (2), lung cancer (3), Merkel cell carcinoma (4), and cell lines derived from head and neck tumors (5, 12). The aberrant FHIT transcripts mostly lack exons 5, 6, and 7, although we have sequenced FHIT transcripts which, in addition to the exon loss described above, also lack exons 4 and/or 8. The mechanism(s) responsible for the formation of the aberrant FHIT transcripts are unknown, but may reflect DNA deletions/rearrangements in this region of the genome. Using Southern blot analysis (Fig. 4), we have shown that the *FHIT* gene is structurally altered in three of the five cervical cell lines that display aberrant FHIT transcripts by RT-PCR. The HT3 cell line displayed a normal pattern for the Southern blot, despite the presence of aberrant transcripts by RT-PCR. However, there could be FHIT genomic alterations not detected by this analysis. A more detailed examination of the *FHIT* gene using a wider array of restriction enzymes combined with exon-specific probes may reveal other abnormalities. Although HeLa displayed a structurally altered gene by Southern analysis, our RT-PCR assay indicated the presence of normally sized FHIT transcripts. Two recent studies reported that the pattern of FHIT expression was altered in HeLa cells, and there was evidence suggesting that the gene was structurally altered (13, 14).

We conclude that the high incidence of abnormalities of the *FHIT* gene transcript and the presence of *FHIT* gene alterations in cervical cancer cell lines (compared to ovarian and endometrial cell lines) suggest that this gene may be altered in cervical cancer tissue. Our analysis of primary cervical tumor tissue would determine whether the *FHIT* gene is altered in this tissue. The *FHIT* gene may provide a useful molecular marker for cervical cancer if this gene is consistently disrupted in cervical cancer, regardless of its role in the tumorigenic process. In fact, although recent evidence indicates that the FHIT protein is a dinucleoside 5′,5′′′-P1,P3-triphosphate hydrolase (15), its exact role in the process of tumorigenesis remains unknown.

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