Loss of FHIT Expression in Cervical Carcinoma Cell Lines and Primary Tumors

David L. Greenspan, Denise C. Connolly, Rong Wu, Rachel Y. Lei, Joshua T. C. Vogelstein, Young-Tak Kim, Jung Eun Mok, Nubia Muñoz, F. Xavier Bosch, Keerti Shah, and Kathleen R. Cho

Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; [I. G., D. C. C., R. W., R. V. L., J. T. C. V., K. R. C.]; Department of Obstetrics and Gynecology, Asan Medical Center and School of Medicine, University of Ulsan, Seoul, Korea [Y-T. K., J. E. M.]; International Agency for Research on Cancer, Lyon, France [N. M.]; Servei D’Epidemiologia I Registre Del Càncer, Institut Català D’Oncologia, E-08907 L’Hospitalet Del Llobregat, Barcelona, Spain [F. X. B.]; and Department of Microbiology and Immunology, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205 [K. S.]

Abstract

Allelic deletions involving the short arm of chromosome 3 (3p13-21.1) have been observed in cervical carcinomas. Recently, a candidate tumor suppressor gene, FHIT (Fragile Histidine Triad), was cloned and mapped to this chromosomal region (3p14.2). Abnormal FHIT transcripts have been identified previously in a variety of tumor cell lines and primary carcinomas, although their significance and the molecular mechanisms underlying their origin remain incompletely defined. In addition, integration of human papillomavirus DNA has been identified at a fragile site (FRA3B) within the FHIT locus in cervical cancer. These observations motivated us to evaluate FHIT mRNA and protein expression in cervical cancer cell lines, primary cervical carcinomas, and normal tissues. Transcripts of the expected size and sequence were the predominant species in all normal tissues evaluated. In contrast, aberrant FHIT transcripts were readily demonstrated in 6 of 7 cervical carcinoma cell lines and 17 of 25 (68%) primary cervical carcinomas. Northern blot analyses demonstrated reduced or absent FHIT expression in the cervical carcinoma cell lines, particularly those with aberrant RT-PCR products. Immunohistochemical analysis of FHIT expression in cervical tissues revealed strong immunoreactivity in nonneoplastic squamous and glandular cervical epithelium and marked reduction or loss of FHIT protein in 25 of 33 (76%) primary cervical carcinomas. In those cervical cancer cell lines and primary tumors with exclusively aberrant or absent FHIT transcripts by RT-PCR, FHIT protein expression was always markedly reduced or absent. The frequent alterations in FHIT expression in many cervical carcinomas, but not in normal tissues, suggest that FHIT gene alterations may play an important role in cervical tumorigenesis.

Introduction

Worldwide, cervical carcinoma remains the second most common malignancy of women in both incidence and mortality (1). Over the past decade, compelling evidence has been obtained that cervical carcinoma is associated with infection by specific types of HPVs. Molecular studies have begun to elucidate the mechanisms by which HPV infection contributes to cervical tumorigenesis. For example, the E6 and E7 oncoproteins encoded by the high-risk (oncogenic) HPV types have been shown to interact with the tumor suppressor proteins p53 and pRB (2, 3). These protein-protein interactions serve, at least in part, to abrogate cell cycle regulation, particularly the G1 arrest in response to DNA damage (4–7). Hence, cells infected by high-risk HPVs may be more susceptible to the accumulation of mutations than their uninfected counterparts.

Although HPV infection clearly plays an important role in cervical tumorigenesis, several lines of evidence suggest that HPV infection alone is insufficient for cervical tumor development. For example, most high-risk HPV infections do not result in cervical cancer, and in those individuals who do develop carcinoma, there is usually a relatively long period (years) between initial infection and the identification of invasive disease. Thus, other events such as tumor suppressor gene inactivation and oncogene activation are likely to be critical in the pathogenesis of cervical cancer. Several analyses of cervical carcinoma have shown a high frequency of allelic deletions on the short arm of chromosome 3 (3p13-21; Refs. 8–13). Recently, a candidate tumor suppressor gene, FHIT, was identified at chromosome 3p14.2 (14). The FHIT gene encompasses the t(3;8) breakpoint that segregates with familial renal cell carcinoma, and FHIT gene abnormalities have been reported in multiple types of tumor cell lines and primary tumors (14–23). Homozygous deletions affecting FHIT coding sequences have been demonstrated in some tumor types, although most studies have focused on the identification of altered FHIT transcripts using RT-PCR assays. In addition, integration of HPV DNA has been identified at a fragile region (FRA3B) within the FHIT gene in cervical cancer (24). To address the hypothesis that FHIT may be frequently inactivated in cervical cancers, we characterized FHIT mRNA and protein expression in several cervical cancer cell lines, primary cervical carcinomas, and various normal tissues, including normal cervix.

Materials and Methods

Cell Lines and Tissues. All cervical carcinoma cell lines (HT-3, SiHa, C-4 II, CaSki, C-33a, HeLa, and ME-180) were obtained from the American Type Culture Collection and maintained in the recommended media including 10% FBS. Two cell lines (C-33a and HT-3) lack detectable HPV sequences and contain mutant p53 alleles, whereas the remaining cell lines are HPV positive with wild type p53 (25). Primary keratinocytes were cultured from fresh human foreskins and cervix as described previously (26) and maintained in keratinocyte growth medium (Clonetics, Walkersville, MD). Fresh normal tissues (brain, lung, liver, kidney, colon, thyroid, cervix, ovary, and myometrium) were obtained from surgical specimens and histologically verified as normal by analysis of frozen sections. A total of 35 invasive squamous carcinomas of the cervix (34 primary tumors and 1 metastatic lesion [tumor T17]) were obtained from the Surgical Pathology Department of the Johns Hopkins Hospital, the Department of Obstetrics and Gynecology of the Asan Medical Center, and from a previous study conducted in Spain and Colombia (27, 28). Frozen tissue was available from 25 of the 35 tumors. Regions containing at least 70% tumor cells (assessed by frozen section) were used as sources of RNA. Formalin-fixed, paraffin-embedded material was available from 33 of the 35 tumors.

RNA Extraction and cDNA Synthesis. RNA was extracted from the cervical carcinoma cell lines, selected primary cervical carcinomas, keratinocytes, and normal tissues using TRIzol (Life Technologies, Inc., Grand Island, NY) or RNAlater Total RNA Isolation System (Promega Corp., Madison, WI).
RT-PCR and cDNA Sequencing. Amplification of FHIT cDNA encompassing the entire open reading frame was performed using both nested (14) and nonnested (29) RT-PCR strategies as described previously. Primers for nested PCR (SU2, SU1, 3D1, and 3D2) yield expected PCR products of 707 bp, extending from exon 3 to exon 10. Primers for nonnested PCR (MURS and RP2) yield expected PCR products of 747 bp, extending from the terminal portion of exon 2 to exon 10. For nested PCR, the first round of cDNA amplification was performed in 25-µl reactions containing 1–2 µl of first-strand cDNA product, 50 µM dNTPs, 1.25 unit of Taq polymerase, and 0.8 µM of FHIT-specific primers. Initial denaturation at 95°C for 3 min was followed by 25 cycles of 15 s at 94°C, 30 s at 62°C, and 45 s at 72°C, and a final extension of 5 min at 72°C using an Omnigene PCR thermocycler. The amplified products were diluted 20-fold with TE buffer, and 1 µl of diluted product was used in a second round of PCR amplification using nested primers for 30 cycles under the above conditions. The PCR products were evaluated on 2% ethidium bromide-stained agarose gels. Selected PCR products were purified using the Wizard PCR Prep DNA purification kit (Promega), cloned into the pCR2.1 vector, and transformed into One Shot cells using the Original TA cloning Kit (Invitrogen, Oma, UT). Following blue-white selection, plasmid DNA preparations from at least four white transformants per PCR product were each sequenced using the Sequenase version 2.0 sequencing kit (Amer sham Corp., Cleveland, OH). Nonnested PCR reactions were performed in 25-µl reaction volumes using 175 ng of each nonnested FHIT primer, 300 µM of dNTPs, 2.5 units of Taq polymerase (Life Technologies, Inc.), and 2 µl of cDNA. Initial denaturation for 90 s at 95°C was followed by 35 cycles of 95°C for 30 s, 62°C for 60 s, and a final extension at 72°C for 5 min. To exclude RNA degradation as an explanation for the absence of FHIT expression, RT-PCR using GAPDH-specific primers (product of 508 bp) was performed on all RNA samples. The GAPDH amplifications were performed in 25-µl reaction volumes using 0.4 µM GAPDH-specific primers (sense, 5'-GAGAAGTATGACAACAGCCTC-3'; antisense, 5'-AGTGGTCGGTT-GAOGOCAAATG-3'), 200 µM dNTPs, and 1.5 unit of Taq polymerase. Reactions were initially denatured at 95°C for 2 min and then amplified with 30 cycles of 94°C at 30 s, 60°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 5 min.

Northern Blot Analysis. To analyze FHIT mRNA expression in cervical cancer cell lines and primary keratinocytes, 20 µg of total RNA were resolved on 1% agarose-4-morpholinepropanesulfonic acid/formaldehyde gels, transferred to a nylon membrane, and hybridized to a radiolabeled cDNA probe spanning FHIT exons 3–9 in Rapid-Hyb buffer (Amersham). The blot was washed at 65°C in 0.3X SSC/0.1% SDS and then autoradiographed. The blot was then stripped of hybridized probe by washing twice in 0.1X SSC/0.5% SDS at 95°C and subsequently rehybridized to a GAPDH cDNA probe to control for potential loading differences between samples. The size of FHIT mRNA(s) was assessed by comparison to RNA standards run on the same gel as the RNA samples.

Immunohistochemical Analysis of Hif Protein Expression. Formalin-fixed, paraffin-embedded cell pellets or tissues were sectioned at 5 µm onto ChemMate-coated slides (Curtin Matheson Scientific, Pittsburgh, PA) and then deparaffinized in two changes of xylene for 5 min each. Sections were hydrated into dH2O through a series of graded alcohols. Antigen enhancement was performed by steaming the sections at 80°C in sodium citrate buffer (diluted to 1X from 10X heat induced epitope retrieval buffer; Ventana BioTek, Tucson, AZ). Slides were cooled for 5 min and then transferred to a Tech Mate 1000 automated stainer (Ventana-BioTek, Tucson, AZ). Slides were washed three times in phosphate buffer and then incubated with blocking serum (normal goat serum) for 5 mm. Following additional phosphate buffer washes, slides were incubated overnight with primary rabbit polyclonal anti-GST-Fhit antisera (30) at 1:10,000 dilution (antiserum generously provided by Dr. Kay Huebner, Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA). Slides were washed again three times and then incubated with secondary antibody (biotinylated goat anti-rabbit IgG) for 30 min. Slides were treated with hydrogen peroxide for 15 min to reduce endogenous peroxidase activity. Avidin-biotin complex was added to the slides for 30 min, and antibody localization was effected by a 20-min incubation with 3,3'-diaminobenzidine. Finally, slides were washed in dH2O, lightly counterstained with hematoxylin, dehydrated, and then coverslipped using AccuMount (Baxter) mounting medium. For blocking exper-

Fig. 1. RT-PCR analysis of FHIT expression in cervical carcinoma cell lines and primary tissues. A, nested RT-PCR analysis of FHIT cDNAs in cervical carcinoma cell lines and primary foreskin keratinocytes (PFK). The cell lines evaluated are indicated above the lanes; left, 1-µg ladder. Arrowhead, size of the expected 707-bp RT-PCR product. B, nonnested RT-PCR analysis of cervical carcinoma cell lines and PPK as above. Arrowhead, size of the expected 747-bp RT-PCR product. C, nested RT-PCR analysis of FHIT expression in normal tissues. The specific tissues are indicated above the lanes; left, 1-µg ladder. Arrowhead, size of the expected 707-bp RT-PCR product.
LOSS OF FHIT EXPRESSION IN CERVICAL CARCINOMAS

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**Fig. 2.** Schematic diagram of representative transcripts in cervical carcinoma cell lines and selected normal tissues. The structure of the FHIT cDNA is shown at the top, and the structure and size of representative cDNA clones from the cervical carcinoma cell lines and selected normal tissues are shown below. Deleted sequences are represented by gaps, and inserted sequences by triangles. The size of the full-length RT-PCR product in normal cells was either 696 or 707 bp, depending on the presence or absence of 11 bp in the 3′-untranslated region. This sequence variation, thought to represent alternative splicing of uncertain significance, has been observed previously (17).

Results

**Aberrant FHIT cDNAs Are Frequently Identified in Cervical Carcinoma Cell Lines and Primary Tumors.** The predominant FHIT transcript in normal tissues encompasses at least 10 exons (30) and is approximately 1.1 kb based on previous Northern blot studies (14). Using RT-PCR assays, FHIT cDNAs of altered size and/or sequence were identified in six (HeLa, HT-3, SiHa, C-4II, CaSki, and C-33a) of the seven cervical carcinoma cell lines evaluated (Fig. 1, A and B). These findings are in agreement with recent studies of cervical carcinoma cell lines (31); however, we found a minor population of aberrant cDNAs in both HeLa and CaSki cells. Both nested (Fig. 1A) and nonnested (Fig. 1B) PCR strategies yielded similar results, although amplification with the nested strategy was generally more robust. For example, C-4II failed to amplify via the nonnested PCR approach (Fig. 1B) but was shown to contain aberrant cDNAs by the nested approach (Fig. 1A). In contrast to most carcinoma cell lines, the dominant FHIT RT-PCR product detected in primary keratinocytes and all normal tissues examined was of the expected size (Fig. 1C). Only cDNAs of the expected size and sequence were identified in the ME-180 cervical carcinoma cell line. Sequence analysis of representative cDNA clones from the cell lines with FHIT transcript alterations identified a variety of aberrant species that are summarized schematically in Fig. 2. Exon 5 (containing the FHIT translation initiation codon) was often absent, although isolated absence of exon 4 was occasionally seen. Insertions of additional sequence were identified in four of the six cell lines with altered FHIT RT-PCR products. These insertions almost always occurred at the boundary between exons 4 and 5, but the novel sequences were not derived from those at this intron-exon boundary. Three cell lines (HeLa, HT-3, and CaSki) showed RT-PCR products of the expected size and sequence in addition to the aberrant ones. One cell line (C-4II) showed a minor population with absence of exon 5 and insertion of nearly the same number of bp at the intron-exon boundary, such that the cDNAs were of the expected size but were, in fact, aberrant.

Seventeen of 25 (68%) primary cervical carcinomas had no detectable FHIT transcripts or had transcripts of aberrant size in the RT-PCR assay (Fig. 3). The presence or absence of the RT-PCR products was generally reproducible in independent RT-PCR experiments, but the relative abundance of the altered products was often variable. Transcripts of the expected size identified in primary tumors may reflect normal FHIT expression in the neoplastic cells, be derived from normal tissue admixed with the tumor, or be induced in response to the tumor milieu.
from contaminating normal cells in the tumor specimen, or represent products of the expected size with abnormal sequence.

**FHIT mRNA Expression Is Markedly Reduced in Most Cervical Carcinoma Cell Lines.** Because of concerns that the RT-PCR assay might not faithfully reflect the relative abundance of FHIT transcripts, Northern blot studies were undertaken. Expression of FHIT mRNA (~1.1 kb) was readily detected in primary keratinocytes. In contrast, substantially reduced or absent FHIT mRNAs were noted in the cervical carcinoma cell lines (Fig. 4), particularly those cell lines that expressed exclusively aberrant FHIT cDNAs in the RT-PCR assay (C-33a and C-411). A weak, diffuse hybridization signal centered at 4.4 kb was identified equally in all samples in the region of 28S ribosomal sequences. The Northern blot studies of FHIT expression in cancer cell lines indicate that the RT-PCR assay, although useful for characterizing altered FHIT transcripts, may not be suitable for quantitation of FHIT expression levels.

**Expression of Fhit Protein Is Markedly Reduced or Absent in Many Primary Cervical Carcinomas.** Studies to quantitate FHIT mRNA expression in primary carcinomas are hindered by several factors, including lack of sufficient quantities of RNA and concerns regarding RNA quality and contaminating normal cells in the tumor specimen. Therefore, we chose to use immunohistochemical methods to characterize FHIT expression in situ. The specificity of the anti-Fhit antiserum was initially tested on formalin-fixed, paraffin-embedded cell pellets from cultured normal keratinocytes and the cervical carcinoma cell lines ME-180 and C-33a. As noted above, keratinocytes and ME-180 cells express only FHIT transcripts of the expected size, whereas C-33a cells express only aberrant transcripts and have undetectable FHIT expression by Northern blot. The anti-Fhit antiserum strongly stained the cytoplasm of normal keratinocytes and ME-180 cells (Fig. 5A) but failed to detect Fhit protein in C-33a cells (Fig. 5B).

Cocubation with excess purified Fhit protein successfully blocked staining in the ME-180 cells (Fig. 5A, inset). Immunohistochemical analysis of primary cervical tumors and adjacent normal tissues revealed strong cytoplasmic staining of Fhit protein in normal ectocervical squamous epithelium (Fig. 5C) and endocervical glandular epithelium (Fig. 5D), which was also effectively blocked by coincubation with purified Fhit protein (data not shown). Coincubation with similar concentrations of an irrelevant protein (human milk fat globule protein) had no effect on Fhit immunoreactivity (data not shown). Staining in the squamous epithelium was localized primarily in the differentiating layers, with relative sparing of the basal and parabasal epithelial cells. Although some primary tumors showed Fhit staining similar to that seen in normal cervix (Fig. 5E), 25 of 33 (76%) tumors showed marked reduction or absence of Fhit immunoreactivity (Fig. 5F). Immunostains of the primary tumors were evaluated independently by two pathologists, and composite scores (~3 versus >3) were concordant in all but 2 of the 33 cases. These two cases were reviewed and reassigned scores based on consensus opinion. The composite score of immunoreactivity correlated quite well with the RT-PCR data in that Fhit protein expression was always reduced or absent (composite score ≤3) in tumors with exclusively aberrant or absent mRNA by RT-PCR (Table 1). Three tumors with substantially reduced Fhit expression contained only transcripts of the expected size by RT-PCR. There are several possible explanations for these observations including: (a) the immunohistochemical assay may more accurately reflect Fhit expression than the RT-PCR assay; (b) the FHIT cDNAs detected were contributed by nonneoplastic contaminating cells; or (c) the transcripts of expected size have aberrant sequence and fail to encode Fhit protein. Finally, when both full-length and aberrant transcripts were observed in a tumor, significant immunoreactivity (composite score >3, <9) was sometimes seen, suggesting that some primary tumors, like the carcinoma cell lines, may express both full-length and aberrant FHIT mRNAs.

**Discussion**

Cervical carcinomas, as other tumors, likely result from an accumulation of genetic alterations activating oncogenes and inactivating tumor

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**Fig. 3.** RT-PCR Analysis of FHIT expression in primary cervical carcinomas. A, nested RT-PCR analysis of FHIT expression in primary tumors. The specific tumors shown are designated above each pair of lanes in which (+) represents reactions performed in the presence of RT and (−) in the absence of RT. Arrowhead, size of the expected 707-bp RT-PCR product. B, RT-PCR analysis of GAPDH expression in primary tumors. The presence of RT and (−) in the absence of RT. Arrowhead, size of the expected 508-bp RT-PCR product.

**Fig. 4.** Northern blot analysis of FHIT expression in cervical carcinoma cell lines and primary foreskin keratinocytes. The cell lines evaluated are as indicated. Based on comparison to the migration of RNA standards of known size, the FHIT mRNAs were estimated to be approximately 1.1 kb. To control for RNA loading, the blot was rehybridized to a control probe for GAPDH.
suppressor genes and/or genes involved in DNA damage recognition and repair. Although the high-risk HPVs have an important role in cervical cancer, relatively few somatic mutations in cellular genes have been identified. Those alterations that have been identified include amplification of c-myc (32), HER2-neu (33), and an as-yet-unidentified gene(s) in the distal region of chromosome 3q (34). Allelic losses of chromosome 3p have been seen in a number of analyses of cervical cancer (11–13), and frequent 3p deletions have also been observed in the intraepithelial lesions of the cervix that are believed to be precursors of invasive squamous carcinomas (35). The common regions of allelic loss appear to be 3p13–14.3 and 3p13-p21.1 (9, 35, 36). Despite considerable efforts, the tumor suppressor gene(s) targeted by 3p allelic losses in cervical carcinoma have not been identified conclusively. One of the regions of 3p commonly deleted in cervical carcinomas includes the FHIT locus (3p14.2). The FHIT gene encompasses a fragile site (FRA3B) spanning FHIT introns 3–5 (37). Our demonstration of altered FHIT transcripts and/or loss of protein expression in most cervical carcinoma cell lines and over 70% of primary cervical tumors supports FHIT as a candidate tumor suppressor gene targeted by 3p deletions and/or HPV integration into the FRA3B fragile site. Notably, we demonstrated FHIT alterations in both HPV-negative and HPV-positive cervical carcinomas.

Several investigators have reported the presence of altered FHIT transcripts in primary tumors and cell lines derived from many types of tumors, including those of the lung, breast, head and neck, gastrointestinal tract, and skin (14–18, 20–22, 38). However, only FHIT cDNAs were analyzed in these studies, and it has been difficult to
assess the origin of full-length transcripts (tumor versus contaminating normal cells) in the evaluated specimens. Moreover, the relation of the human Fhit protein is not known, but it bears a homology to the tumor suppressors p53 and Rb. Therefore, it could act as a possible tumor suppressor gene and be involved in cervical tumorigenesis. The exact role of Fhit in the pathogenesis of cervical cancer awaits further analysis and assessment of the in vivo function of the protein in normal, preneoplastic, and neoplastic tissues.

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References

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