

Role of 5'-CpG island hypermethylation of the *FHIT* gene in cervical carcinoma

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Objective: The abnormal expression of fragile histidine triad (*FHIT*) gene has been frequently reported in a variety of epithelial malignancies including cervical carcinoma. Furthermore, in a recent study it was proposed that transcriptional inactivation of *FHIT*, as a consequence of aberrant 5'-CpG island methylation, plays an important role in the carcinogenesis of human cervical carcinoma. The authors sought to determine whether abnormal *FHIT* transcription occurs in human cervical carcinoma, and if so, whether this abnormal expression is associated with aberrant 5'-CpG island methylation. In addition, the clinical significance of *FHIT* inactivation was investigated in Korean women with cervical cancer.

Methods: To examine for abnormal transcripts of the *FHIT* gene, quantitative RT-PCR, genomic DNA-PCR and nonisotopic RT-PCR-SSCP analysis were performed using the standard method. The methylation status was determined by methylation specific PCR and bisulfite DNA sequencing.

Results: The *FHIT* gene was down-regulated in 15 of 58 (25.9%) cervical carcinomas. *FHIT* promoter hypermethylation was detected in 15 of 15 (100%) abnormally expression in cervical carcinomas. Bisulfite DNA sequencing confirmed these findings and a significant correlation was found between CpG site hypermethylation and low *FHIT* expression. However, no significant correlation was found between reduced *FHIT* expression and clinicopathological characteristics.

Conclusion: In this study, *FHIT* inactivation in cervical cancer was found to be strongly correlated with 5'-CpG island hypermethylation rather than a genetic alteration. Furthermore, no significant relation was found between a lack of *FHIT* expression and the prognostic factors of cervical cancer in our Korean cohort.

Key Words: Cervical carcinoma, *FHIT* gene, Hypermethylation, Bisulfite-DNA sequencing

INTRODUCTION

Worldwide, cervical cancer is the leading gynecological malignancy, and in 2002 was found to be the 5th most common malignant disease in Korean women.¹ It is well known that human papilloma virus (HPV) is the major causative agent,² though many patients infected with HPV remain stable. Thus, various other epigenetic events, such as CpG island hypermethylation, are likely to be involved in the genesis of cervical cancer. Furthermore, the hypermethylation of cytosine in normally unmethylated CpG-rich

sequences, called CpG islands, is a frequent epigenetic event in cancers of the breast, prostate, liver, thyroid, oral squamous cell carcinoma, and others.³⁻⁵

The short arm of human chromosome 3 commonly contains chromosomal abnormalities in cancer patients,⁶ and chromosomal rearrangements are frequently observed at 3p25, 3p21.3, 3p14.2 and 3p12.⁷ In particular, the 3p14.2 region is of interest because it contains *FRA3B*, the most active fragile site in the human genome, which contains the fragile histidine triad (*FHIT*) gene. Furthermore, *FHIT* has been identified as a candidate tumor suppressor gene,⁸ and although its effect on the cell cycle is unclear, it has been reported that reexpression of *FHIT* in a variety of human cell lines results in growth inhibition and apoptosis induction.⁹ 5'-CpG island methylation of *FHIT* has been documented in prostate, breast, esophageal, and lung carcinomas, which suggests that it participates in tumorigenesis.¹⁰⁻¹³ In particular, *FHIT* was found to be hypermethylated in 40-50% of cervical cancer tissue.¹⁴ *FHIT* abnormalities include loss of

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heterozygosity, homozygous deletions, and the insertions of other sequences. However, point mutations in *FHIT* are rare and some homozygous deletions may only affect introns.^{15,16}

In this study, we aimed to elucidate the mechanism that underlies the loss of *FHIT* expression in cervical carcinoma by measuring 5'-CpG island DNA methylation status in *FHIT*, and by investigating the nature of the relationship between *FHIT* hypermethylation and clinicopathological characteristics.

MATERIALS AND METHODS

1. Samples and clinical data

Fifty eight primary cervical carcinomas and seven normal cervical epithelial samples were surgically collected during the period 1996-2002 at Kyung Hee University Hospital (Seoul). All normal cervical specimens were obtained from patients with no evidence of cancer during surgery for benign gynecologic conditions. Tissue specimens were snap-frozen immediately in liquid N₂ and stored at -70°C until required. Total cellular RNA was extracted using the single step method and genomic DNA was later extracted from the same cells. Clinical information was retrospectively obtained from medical records, and included patient age, tumor stage, date

of diagnosis, histologic subtype, pathologic results, and follow-up data.

Tumor stages were assigned according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer. The clinicopathological characteristics of samples are given in Table 1. Four of the tumors were stage IA, 37 stage IB, and 17 stage II. Histologically, 50 of the 58 tumors were squamous cell carcinomas, 4 were adenocarcinomas, and 4 were adenosquamous carcinomas.

2. Quantitative RT-PCR

One microliter of extracted total cellular RNA was converted to cDNA by reverse transcription using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 1 : 4 diluted cDNA (12.5 ng/50 µl of PCR reaction mix) achieved the logarithmic amplification phase after 24-36 cycles using primers for *FHIT* (sense: 5'-GAGAAATCCACTGAGAACAGTC-3' (Exon 2), antisense: 5'-ATCAGGACGCAGGTCATGGAAG-3 (Exon 6), PCR product length : 354 bp), and for the GAPDH endogenous standard. Optimal PCR cycling conditions involved 26-32 amplification cycles at 95°C for 1 min (denaturation), 58-64°C for 0.5 min (annealing), and 72°C for 1 min (extension) in a reaction buffer containing 1.5 mM MgCl₂ (PCR buffer II, Perkin-Elmer Corp., Norwalk, CT).

3. Quantitative genomic PCR analysis

Genomic DNA was extracted from the same tissue samples after RNA extraction. 200 ng of genomic DNA was to the *FHIT* amplification using an intron-specific primer pair (Sense: 5'-TCAACTCTCTGGAGTTCAGTGG-3' (Intron 5), Anti-sense: 5'-GGACAGTAGGGTTGCCCTGCAT-3' (Intron 6), PCR product length : 470 bp).

RT-PCR products were confirmed by Southern hybridization, 10 µl of RT- and genomic PCR products were resolved on 2% agarose gels. Quantitation was achieved by densitometric scanning of ethidium bromide stained gels. Absolute area integrations of the curves representing each specimens were compared after adjusting for *GAPDH*, an endogenous expression standard gene. Integration and analysis were performed using the Molecular Analyst software program (Bio-Rad, Hercules, CA). Quantitative PCR was repeated at least three times per specimen and mean were calculated.

4. Non-isotopic RT-PCR-SSCP analysis

To screen for the presence of somatic mutations in *FHIT*, RT-PCR-SSCP was performed. The *FHIT* transcript was amplified with seven sets of primers designed to cover the entire coding region of the gene (sequences of the primers used are available on request). PCR products of more than 300 bp were digested with endonucleases to increase the sensitivity of SSCP. PCR products (20 µl) were mixed with 10 µl of 0.5 N NaOH, 10 mM EDTA, and 15 µl of denaturing

Table 1. Association between abnormal *FHIT* mRNA expression and clinicopathologic parameters

Parameters	Case No.	<i>FHIT</i> low expression No. (%)	p-value
FIGO stage			
I	41	13 (31.7)	
II	17	2 (11.6)	NS
Pathology			
Squamous cell carcinoma	50	12 (34.0)	
Adenocarcinoma	4	1 (25)	
Adenosquamous carcinoma	4	2 (50)	NS
Differentiation			
Well	11	4 (36.4)	
Moderate	42	10 (23.8)	
Poor	5	1 (20)	NS
Tumor size (cm)			
≤4	44	11 (25)	
>4	13	3 (23.1)	NS
Invasion depth			
≤inner 1/3	15	3 (20)	
Mid 2/3	23	6 (26.1)	
≥outer 2/3	20	6 (30.0)	NS
LN metastasis*			
Negative	40	9 (22.5)	
Positive	17	6 (35.3)	NS
LVS invasion [†]			
Negative	48	11 (22.9)	
Positive	10	4 (40)	NS

* LN; lymph node, † LVS; lymphovascular space

loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). After heating at 95°C for 5 min, samples were loaded into wells pre-cooled to 4°C and run using 8% non-denaturing acrylamide gels containing 10% glycerol at 4-8°C and 18-22°C.

5. Bisulfite DNA sequencing analysis

Genomic DNA (1 µg) in a volume of 50 µl was denatured with NaOH (final concentration 0.3 M), and 30 µl of 10 mM hydroquinone and 520 µl of 3M sodium bisulfite (pH 5.0) were added and incubated at 55°C for 16-20 h. DNA samples were purified using the Wizard DNA clean-up system (Promega Corp., Madison, WI), again treated with NaOH at 37°C for 15 min, precipitated with ethanol, and resuspended in distilled water. Bisulfite-modified DNA (50 ng) was then subjected to PCR amplification of the *FHIT* intron 1 region using the following primer sets; *FHIT*-MS1 (sense; 5'-GGAGGTAAGTTTAAGTGGAA-3') and *FHIT*-MS2 (antisense; 5'-CCCACCCTAAAACCTCTTTT-3'). The PCR products obtained were cloned into pCRII vectors (Invitrogen, Carlsbad, CA) and 5 clones of each specimen were automatically fluorescence-based DNA sequenced to determine methylation status.

6. Methylation-specific PCR analysis

PCR was performed with the methylation-specific primers *FHIT*-MS-1 (sense, 5'-TGGGGCGCGGGTTTGGGTTTTT-ACGC-3') and *FHIT*-MS-2 (antisense, 5'-CGTAAACGAC-GCCGACCCCACTA-3') and the unmethylation-specific primers *FHIT*-UMS-1 (sense, 5'-TTGGGGTGTGGGTTTGGG-TTTTTATG-3') and *FHIT*-UMS-2 (antisense, 5'-CATAAACAA-CACCAACCCCACTA-3') using 200 ng of the bisulfite-modified genomic DNA as a template for 38 cycles of 95°C for 1 min, 60-63°C for 1 min, and 72°C for 1 min. PCR products (10 µl) were then resolved on 2% agarose gels.

RESULTS

1. Loss of *FHIT* mRNA expression in cervical carcinoma

The mRNA expression of *FHIT* was examined in 58 human cervical carcinomas and 7 noncancerous cervical tissues. As shown in Fig. 1, *FHIT* transcripts were easily detected in noncancerous cervical tissue samples, at levels of 0.72-0.92 (mean; 0.82 ± 0.07). In contrast, the mRNA expression statuses of *FHIT* in cervical carcinomas were significantly lower (range; 0.18-0.92, mean; 0.58 ± 0.22 , p-value < 0.05).

Levels of less than half the normal mean (i.e., < 0.41) were defined as abnormally low. Accordingly, 25.9% (15 of 58) of primary tumors were deemed to express *FHIT* at abnormally low levels. Nevertheless, *FHIT* expression showed no statistical correlation with clinicopathologic parameters as previously described¹⁷ (Table 1).

2. Absence of allelic deletion of the *FHIT* gene in cervical carcinoma

To elicit whether loss of or reduced *FHIT* mRNA expression in cervical carcinomas is associated with the allelic deletion of *FHIT*, we examined *FHIT* levels using quantitative genomic PCR. None of the tumors, including the 15 with abnormally low expression, showed a detectable reduction in *FHIT* level, which suggests that genomic deletion of *FHIT* is infrequent and not associated with the abnormal downregulation of *FHIT* mRNA in human cervical carcinoma.

3. Tumor-specific downregulation of *FHIT* by the hypermethylation of promoter CpG sites

Methylation-specific PCR (MSP) of *FHIT* promoter sequences was used to determine the overall frequency of *FHIT* hypermethylation in tumors. Methylation-specific primers (*FHIT*-MS-1 and *FHIT*-MS-2) and unmethylation-specific primers (*FHIT*-UMS-1 and *FHIT*-UMS-2) were designed, and methylation was detected in all 15 tumor cell lines with no or diminished *FHIT* expression. Moreover, it was not found in

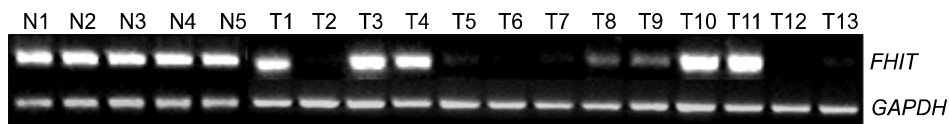


Fig. 1. Reduced expression of *FHIT* mRNA in human cervical cancer tissues (quantitative RT-PCR analysis). N; normal cervix tissue, T; cervical cancer tissue.

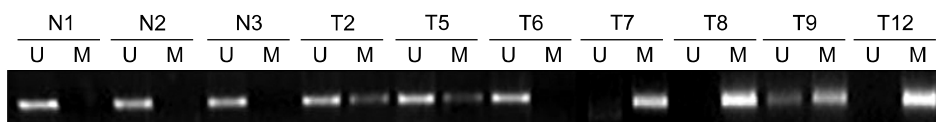


Fig. 2. *FHIT* 5'-CpG island hypermethylation in human cervical cancer (methylation-specific PCR analysis). N; normal cervix tissue, T; cervical cancer tissue, U; unmethylation-specific PCR, M; methylation-specific PCR.

any of the seven noncancerous cell lines that expressed *FHIT* at normal levels (Fig. 2).

4. Aberrant hypermethylation at CpG sites in *FHIT* promoter

To explore the relationship between aberrant CpG methylation and gene silencing, we performed bisulfite DNA sequencing analysis of *FHIT* promoter and analyzed the

methylation status 13 CpGs located in its 5' proximal region. The sequence (intron1) spanning these 13 CpG sites was amplified by PCR using sodium bisulfite-modified DNA as a template, and 5 PCR clones were sequenced to determine methylation frequencies at individual CpG sites (4-5 clones; complete methylation, 2-3 clones; partial methylation, 0-1 clones; unmethylation) (Fig. 3).

A tight correlation was observed between gene silencing and

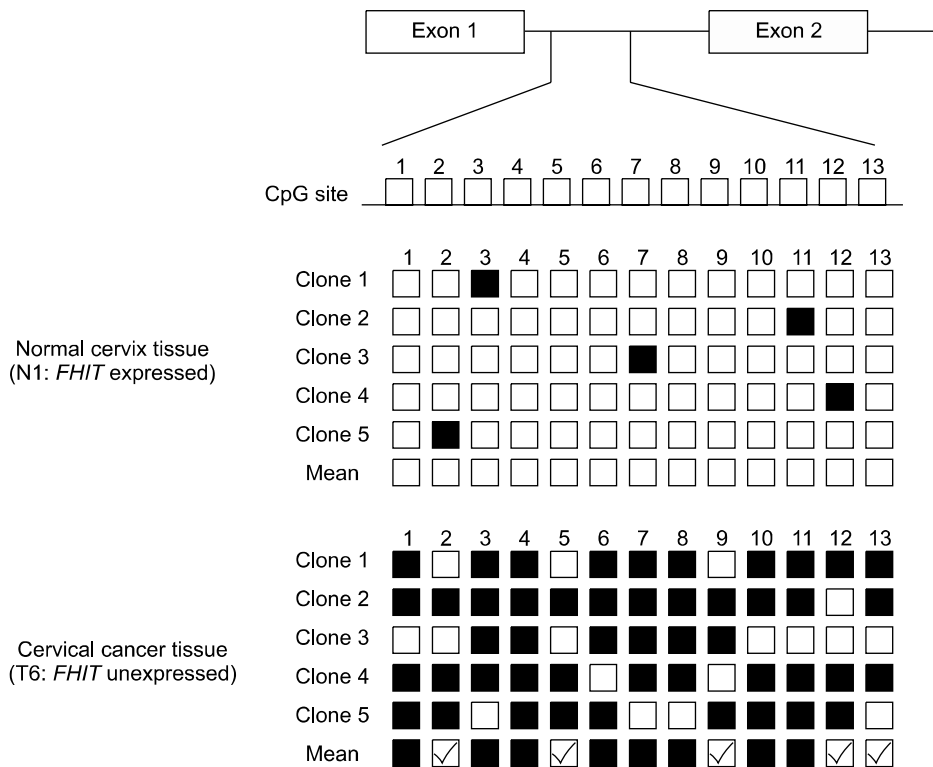


Fig. 3. *FHIT* 5'-CpG island hypermethylation in human cervical cancer (sodium bisulfite DNA sequencing analysis). PCR products were cloned, and 5 plasmid clones were sequenced (■ methylated CpG; □ unmethylated CpG). methylation status of 13 CpGs in *FHIT* promoter. mean methylation status was determined from the number of alleles containing a methylated CpG at each position (■ complete methylation; 4-5 clones, ☑ partial methylation; 2-3 clones, □ unmethylation; 0-1 clone).

CpG site	1	2	3	4	5	6	7	8	9	10	11	12	13	mRNA expression
N1	□	□	□	□	□	□	□	□	□	□	□	□	□	0.8
N2	□	□	□	□	□	□	□	□	□	□	□	□	□	0.9
N3	□	□	□	□	□	□	□	□	□	□	□	□	□	0.9
T2	☑	☑	■	☑	☑	■	■	■	☑	☑	☑	■	☑	0.5
T5	☑	☑	☑	■	☑	☑	■	☑	☑	☑	■	■	☑	0.5
T6	■	☑	■	■	☑	■	■	■	☑	■	■	☑	☑	0.2
T7	■	■	■	☑	■	■	☑	■	■	■	■	■	■	0.2
T8	■	☑	■	■	☑	■	■	■	☑	■	■	☑	☑	0.3
T9	■	■	☑	■	■	■	■	☑	☑	■	■	■	■	0.2
T12	■	■	■	☑	■	■	☑	■	☑	■	■	■	■	0.3
T13	■	■	■	☑	■	☑	■	■	■	■	■	■	■	0.3
T16	□	□	□	□	☑	□	□	□	□	□	□	□	☑	0.9
T19	■	■	■	■	☑	☑	■	■	■	☑	☑	■	☑	0.4

Fig. 4. Methylation status of the *FHIT* CpG Island in cervical cancer (correlation between CpG site hypermethylation with low *FHIT* expression). N; normal cervical tissue, T; cervical cancer tissue.

methylation of the 13 CpGs. All 13 CpGs were partially or completely methylated in the 15 carcinoma tissues showing abnormal expression, whereas none of the CpGs were methylated in the 7 normal cervical tissues. Furthermore, in carcinoma tissues showing normal expression, these 13 CpGs were partially methylated or non-methylated. In addition, completely methylated CpG sites were more frequently observed when mRNA expressions were lowest (Fig. 4), which indicated that methylation extent correlates with mRNA expressional status.

5. Absence of *FHIT* mutations in cervical cancer

RT-PCR-SSCP was performed on all 58 primary carcinomas to evaluate the mutational statuses of *FHIT*. The entire coding regions of *FHIT* transcripts were amplified using seven sets of exon-specific primers. To improve mutation detection sensitivity, the same RT-PCR products were digested using a different restriction endonucleases and SSCP was performed using two different running conditions. However, no mutations leading to an amino acid substitutions or frameshift were found in the *FHIT* transcripts expressed, thus indicating that somatic mutations of *FHIT* are infrequent in cervical cancer.

DISCUSSION

Promoter methylation is the primary epigenetic alteration found in the human genome, and is believed to play an important role in tumorigenesis. It has been well demonstrated that hypermethylations of CpG-rich promoter or exonic regions are strongly associated with transcriptional silencing, and that CpG islands are more methylated in cancerous tissues than in non-CpG island regions. Furthermore, hypermethylation at CpG islands in transcription regulatory regions are known to lead to the epigenetic inactivations of tumor suppressor genes during tumorigenesis in man.¹⁸

FHIT is a tissue-specific tumor suppressor gene,¹⁹ and its inactivity correlates with the occurrence and development of cancer, especially epithelial cancer.^{8,19} In addition to genetic alterations, promoter methylation of CpG islands has been reported to silence *FHIT*.²⁰ In previous reports, we suggested that *FHIT* is inactivated in cervical carcinoma by epigenetic (promoter methylation) alterations rather than genetic mutations (deletions or chromosomal rearrangements).^{17,21}

To determine the reason for diminished *FHIT* expression in cervical carcinoma, we investigated the methylation status of the *FHIT* 5'-CpG island using methylation-specific PCR and bisulfate DNA sequencing. Several studies have demonstrated that only the region situated between nucleotides 195 and 283 (GenBank, Accession No. U76263) of the 5'-CpG island of *FHIT* is highly methylated in primary tumors.^{12,22} Therefore, we used primers specific for methylated and unmethylated DNA to amplify the *FHIT* 5'-CpG island region

using methylation-specific PCR.

To investigate the connection between hypermethylation of the *FHIT* 5'-CpG Island and loss of *FHIT* expression we analyzed levels of *FHIT* expression in selected samples with methylated and unmethylated *FHIT* 5'-CpG islands. High levels of *FHIT* expression were observed in all normal tissues and in tumor tissues with a unmethylated *FHIT* 5'-CpG island. Conversely, *FHIT* expression was significantly depressed in all tumors with a hypermethylated *FHIT* 5'-CpG Island. These findings demonstrate that *FHIT* 5'-CpG island hypermethylation and reduced *FHIT* expression are related. Furthermore, our findings suggest that *FHIT* 5'-CpG island hypermethylation is an important mechanism of *FHIT* inactivation in cervical carcinoma.

In the present study, *FHIT* expression was downregulated in 25.9% (15/58) of invasive cervical carcinomas, and promoter hypermethylation was detected in all 15 cervical carcinomas showing abnormally low *FHIT* expression. Whereas other studies have reported *FHIT* downregulation in 43% to 71% of cervical carcinomas.^{23,24}

Several studies which examined different tumor types have shown that *FHIT* expressional loss and *FHIT* 5'-CpG island hypermethylation are associated with an advanced tumor stage, poorer overall survival and prognosis, and with tumor recurrence.²⁵⁻²⁹ A study of cervical cancer revealed that low *FHIT* expression is associated with a poorer prognosis in advanced cervical carcinoma,^{30,31} and another study on cervical carcinoma found that *FHIT* downregulation is correlated with lymph node metastasis and tumor invasion.³² As cancer stage and histologic grade increases, so does the rate of methylation. By using this as a potential diagnostic and treatment tool, cervical cancer may be detected more readily and treated more efficiently.³³

In the present study, we were not able to determine whether a statistically significant correlation exists between *FHIT* 5'-CpG island methylation status and tumor stage or tumor histological grade, because of the size and make-up of our patient group (no precancerous lesions were included). A more detailed study on a cohort with a broader disease spectrum is required to resolve this issue. Although we did not find any significant correlation between *FHIT* methylation status and clinicopathological characteristics of the cervical carcinoma patients, a perfect correlation was found between *FHIT* mRNA expression and hypermethylation status. Assays for hypermethylation status is representative biomarker of *FHIT* expression. However, we suggest caution in its use as a functionally relevant biomarker for cervical carcinoma.

In summary, our results shown that all tumors found to have a methylated *FHIT* 5'-CpG island showed a reduction in *FHIT* expression, and that *FHIT* expression levels were normal in tumors with a unmethylated *FHIT* 5'-CpG island and in normal tissues. These results suggest that *FHIT* 5'-CpG island hypermethylation underlies *FHIT* inactivation in cervical carcinoma.

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