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RAPID COMMUNICATION

Fragile histidine triad gene alterations are not essential for hepatocellular carcinoma development in South Korea

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Abstract

AIM: To establish the role of FHIT in the pathogenesis hepatocellular carcinoma (HCC).

METHODS: We examined genomic alterations, as well as, mRNA and protein expression patterns from the *FHIT* gene, in 48 surgically resected hepatocellular carcinoma (HCC) tissues. Additionally, *p53* mutations were analyzed.

RESULTS: Aberrant *FHIT* transcripts were detected in 11 of 48 surrounding non-tumor liver tissues and 27 of 48 HCC samples (22.9% *vs* 56.3%, $P = 0.002$). No point mutations were identified within the open reading frame region of *FHIT*. Loss of heterozygosity (LOH) of the *FHIT* locus was detected in 4 of 42 informative cases for D3S1300, and 3 of 29 informative cases for D3S1313. Reduced expression of *FHIT* protein (Fhit) was observed in 8 (16.7%) of 48 HCC samples, with complete loss of Fhit in only 1 case. There were no associations with abnormal transcripts, LOH, and Fhit expression. *p53* mutations were identified in 9 of the 48 HCC cases. However, none of the cases displayed a G to T transversion at *p53* codon 249.

CONCLUSION: Aberrant *FHIT* transcripts were more common in HCC tissues as compared to non-cancerous liver tissues. However, Fhit expression was lost or reduced in a minor fraction of HCC tissues, while it was strongly expressed in non-cancerous liver tissues. Therefore, our study suggests that *FHIT* plays a role in relatively few HCC cases in South Korea.

Key words: Fragile histidine triad; Aberrant transcripts; Microsatellite instability; Protein expression; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common cancer worldwide and the fourth leading cause of cancer-related deaths. The number of new cases is estimated as more than 500 000 per year, accounting for 4% of all newly diagnosed cancers[1]. More than 80% of HCC cases occur in developing countries, especially in South-East Asia and sub-Saharan Africa, but the incidence is increasing in economically developed regions, including Japan, Western Europe, and the United States[1-2]. The overall prognosis of HCC is poor, because many patients at presentation are already in an advanced and unresectable state, and will have a median survival time of less than 6 mo[3]. High mortality may be partially attributable to the fact that the nanocapsular part of the liver lacks sensory fibers, leading to symptoms presentation only in the advanced HCC[3]. Therefore, only a small proportion of patients are eligible for liver resection, which results in a 5-year survival rate of about 40%[4]. However, even following surgical resection, recurrence rates can be as high as 50% at 2 years[4].

Hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to the malignant transformation of hepatocytes.

Numerous genetic and epigenetic alterations contribute to the activation of carcinogenic pathways in HCC[4]. Whereas most studies on the mechanisms of tumorigenesis have focused on genetic changes, various epigenetic changes have been increasingly identified in HCC[4]. The short arm of human chromosome 3 is one of the most common sites of chromosomal abnormality in malignant diseases[5-8]. One candidate is the fragile histidine triad (*FHIT*) gene, located on 3p14.2, spanning the FRA3B common fragile site[9-10]. The *FHIT* gene is composed of 10 exons encompassing 1.8 Mb genome regions, of which only exons 5 to 9 code for protein. It encodes a small mRNA of 1.1 kb, and a small protein of 16.8 kDa[9]. Studies on the functional aspects of *FHIT* have been reported that this gene is a bona fide tumor suppressor gene[11]. The *FHIT* gene and its protein have may be involved in the regulation of cell proliferative and apoptotic processes[11]. Down-regulation of *FHIT* inhibits apoptosis and *FHIT* protein (Fhit) interacts with a number of key proteins involved in cancer progression, including *p53*[11-12]. Coexpression of *FHIT* and *p53* synergistically inhibits the proliferation of various tumor cell lines. These synergistic effects may occur because of stabilization of *p53* related to Fhit-mediated downregulation of MDM2[12]. Altered transcripts and allelic loss of the *FHIT* gene are frequently identified in premalignant and malignant lesions of various tumors[13-18]. Moreover, loss or reduction of Fhit expression has been found in most tumors including HCC[10-14,19-22].

To establish the role of *FHIT* in the pathogenesis of HCC, we examined genomic alterations, as well as, mRNA and protein expression in surgically resected HCCs and their associated non-cancerous surrounding liver tissues. We also investigated the possible associations between *FHIT* abnormalities and *p53* mutations.

MATERIALS AND METHODS

Patients

HCC samples and their corresponding non-cancerous liver tissues were obtained from 48 patients who had undergone surgical resection at Ulsan University Hospital in South Korea. Written informed consent was obtained from all patients participating in this study, and the study was approved by the Institutional Review Boards at Ulsan University Hospital. All cancer samples were obtained from within the tumor. Matching surrounding non-cancerous liver tissues were obtained as far as possible from the tumors. All specimens were frozen immediately after surgical resection, and stored at -70℃. HCC diagnosis was based on histologic confirmation or elevated serum AFP (> 400 ng/mL) with radiologic findings, or at least two coincident radiologic findings (contrast-enhanced dynamic computer tomography (CT), contrast-enhanced dynamic magnetic resonance imaging (MRI), and Doppler ultrasonography compatible with HCC.

Postoperative follow-up included a dynamic CT/MRI study every 3 mo, and laboratory testing of the serum AFP level every 1 to 3 mo at our outpatient clinic. In the case of suspected HCC recurrence, further examinations, including angiography and lipiodol CT, were performed. If necessary, ultrasound-guided biopsy was conducted to confirm the diagnosis. Bone scintigraphy or chest CT was performed when clinically indicated. The follow-up period was defined as the interval from the date of surgical resection of HCC until the date diagnosis of the recurrence, or the date of death, or the end of follow-up.

Reverse transcription polymerase chain reaction (RT-PCR) and sequencing of FHIT gene transcripts

Total RNA and DNA were isolated from the HCCs and corresponding non-cancerous tissues, using the TRIZOL reagent (Gibco BRL Life Technologies, Gaithersburg, MD, USA). cDNA was synthesized by reverse transcriptase from 1 μg of total RNA. One ul of the RT reaction was used to amplify the FHIT cDNA as described by Ohta *et al*[9]. To amplify FHIT exons 3-10, a nested PCR was carried out in 10-μL final volume with 30 ng of primers (5U2-3D2 and 5U1-3D1, according to Ohta *et al*[9]. The first round of PCR amplification was performed in 10 uL of reaction mixture comprising 1 μmol/L primers 5U2-3D2 and 5U1-3D1, 200 μmol/L each dNTP, 1 × PCR buffer, 2.5 U of ampliTaq, and 0.5 μL of the synthesized cDNA mixture under the following reaction conditions: denaturation for 10 min at 95℃, 34 cycles of 30 s at 95℃, 30 s at 56℃, and 45 s at 72℃, and a final extension step for 10 min at 72℃. After 10-fold dilution of the amplified product 10-fold in DW, 2 μL of aliquots were subjected to a second round of PCR amplification using nested primers, 5U1 and 3D1, under the above conditions. Each nested RT-PCR assay was repeated at least twice with the original extracted RNA for confirmation. All reactions were performed at least twice and the integrity of the RNA samples verified. To confirm the presence of mRNA and rule out nonspecific amplification, 25 cycles of ®-*actin* cDNA were preformed after RT, both with and without the RT enzyme. PCR products were directly sequenced using primer 5U1 and 3U1 with an automated Applied Biosystem Model 3730 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). For sequence analysis of abnormal *FHIT* transcripts, the PCR products were resolved in 1.5% ethidium bromide agarose gel. Bands were excised from gels, and PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA). PCR products excised from gels were directly sequenced using the sequencing primers described by Zekri *et al*[18].

Microsatellite polymorphism analysis

To investigate the allelic loss of *FHIT* gene, a PCR-based approach was performed utilizing primers that amplify two polymorphic microsatellite markers internal to and flanking the *FHIT* gene. The microsatellite marker D3S1300 is located in intron 5 of the *FHIT* gene, whereas marker D3S1313 is slightly telomeric to *FHIT*. The 10 μL PCR mixture contained 1 × Gold Buffer, 0.8 μL 25 mmol/L $MgCl_2$, 0.8 μL 10 mmol/L

dNTP, 1 μL 5 μmol/L concentrations of each primer pair, 0.5 μL 0.25 unit Amplitaq Gold, and 2 μL of the 10 ng extracted DNA. 1 μL of diluted PCR product was mixed with 9 μL of loading buffer (formamide: Ro × 500 size standard, 1:39). The mixture was denatured at 95℃ for 5 min, chilled on ice, and loaded on an ABI PRISM 3100 automatic sequencer. The data were analyzed using the GeneScan and Genotyper software (Perkin-Elmer/Applied Biosystems). Constitutional homozygosity was regarded as uninformative. For informative cases, allelic loss was scored as positive if the signal of one allele was lost or reduced to at least 50% in tumor DNA, compared with the corresponding normal allele. All samples were run twice to confirm the presence or absence of allelic imbalance.

Western blotting analysis

Protein concentration was measured using the BCA protein assay (Pierce, Rockford, IL). Ten micrograms of lysate protein was separated by SDS-PAGE using a 12% polyacrylamide gel and electroblotted onto a Hybond ECL (Amersham Pharmacia Biotech). After blockage of nonspecific binding sites for 1 h with 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated at room temperature for 2 h with rabbit anti-FHIT antibody (Zymed Laboratories, South San Francisco, CA, USA) at a dilution of 1:500. The membrane was washed three times with TPBS, incubated further with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (New England Biolabs, Beverly, MA, USA) at room temperature, and then washed three times with TPBS. Membranes were developed with the ECL chemiluminescence system (Amersham Pharmacia Biotech). The intensity of Fhit expression in HCC tumors was compared with that in non-cancerous surrounding liver tissues.

p53 mutation analysis

The primers used were oligonucleotides complementary to the sequence flanking the exon/intron junctions of exons 5-9. The sequence of the primers is as follows: exon 5, 5'-CTGACTTTCAACTCTG-3' (forward) and 5'-AGCCCTGTCGTCTCT-3' (reverse); exon 6, 5'-CTC TGATTCCTCACTG-3' (forward) and 5'-ACCCCAGTTGCAAACC-3' (reverse); exon 7, 5'-TGCTTGCCACAGGTCT-3' (forward) and 5'-ACAGCAGGCCAGTGT-3' (reverse); exon 8, 5'-AGGACCTGATTTCCTTAC-3' (forward) and 5'-TCTGAGGCATAACTGC-3' (reverse); and exon 9, 5'-TATGCCTCAGATTCACT-3' (forward) and 5'-ACTTGATAAGAGGTCC-3' (reverse). The same primers sets were used for DNA sequencing. PCR was carried out in a 20-μL reaction contained; 50 ng DNA, 10 × PCR buffer, 200 μmol/L each of dNTP, 5 pmol of each primer and 1 U of Taq DNA polymerase. PCR conditions were as follows: 95℃ (10 min) for 1 cycle, 95℃ (40 s), 63℃ (40 s; for exons 4, 5, and 7-9) or 67℃ (40 s; for exon 6), 72℃ (40 s) for 40 cycles, and a final extension step of 72℃ (10 min). PCR products were separated by electrophoresis and visualized with 1.5% ethidium bromide. Samples without the DNA template were included in all assays as negative controls. PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA), and they were sequenced by the dideoxychain termination method with the Big Dye Terminator cycle sequencing kit (Perkin-Elmer Corporation, Foster City, CA, USA). Cycle sequencing was performed for 25 cycles of denaturation (96℃, 30 s), annealing (50℃, 15 s), and extension (60℃, 4 min), according to the Big Dye Terminator protocol. After spin column purification with Centri-Sep columns (Perkin-Elmer Corporation), samples were analyzed with the automated Applied Biosystem Model 3730 DNA sequencer (Perkin-Elmer, Foster City, CA, USA).

Statistical analysis

All continuous variables were compared using the Mann-Whitney test, or the one-way analysis of variance (ANOVA). Categorical variables were compared using the Fisher's exact test. All data were analyzed using the statistical package SPSS (version 14.0: SPSS Inc., Chicago, IL, USA). In all cases, a 2-tailed *P* value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The baseline characteristics of the 48 patients were shown in Table 1. The patients included 35 men and 13 women with a mean age of 50 years (range 28-71 years). HBV was the most common etiology, accounting for 38 (79%) of the 48 cases. Antibody to HCV was identified in three cases. Seven patients were negative for HBV and HCV. Alpha-fetoprotein (AFP) level ranged from 1.0 to 240 000 ng/mL with a median level of 202 ng/mL. Twelve (25%) of the 48 patients had normal AFP levels (< 11 ng/mL), whereas 20 (41.7%) patients had AFP levels of > 400 ng/mL. Preoperative diagnosis of HCC was based on histologic confirmation in 28 cases, or elevated serum AFP (> 400 ng/mL) with radiologic findings, or at least two coincident radiologic findings compatible with HCC in the remaining 20 cases. In 22 of the 48 patients, cirrhosis was observed in the surrounding non-cancerous liver tissue, and chronic hepatitis was identified in parenchyma surrounding the tumors in the remaining 26 cases. According to the Child-Pugh classification at the time of operation, 19 patients were class A (CPT 5-6), 3 were class B. Based on UICC TNM staging[23], 5 patients were stage Ⅰ, 41 stage Ⅱ, and 2 stage Ⅲ. Small HCC was defined as a single tumor < 5 cm, or 2-3 tumors < 3 cm without invasion of major veins larger than sub-segmental branches. These criteria are similar to those applied to define early stage HCC in the BCLC (Barcelona Clinic Liver Cancer) staging scheme[24]. Tumors exceeding these limits were regarded as advanced HCC. At initial diagnosis, 26 patients had small HCC, while 22 patients were advanced HCC. The mean tumor size was

Table 1 Baseline characteristics of 48 patients with HCC

Characteristics	
Mean age (yr)	50 (20-71)
Sex (M:F)	35:13
Etiology of liver disease	
HBV	38
HCV	3
Non-HBV, Non-HCV	7
Tumor size	
⩽ 3 cm	11
3-5 cm	15
> 5 cm	22
Mean (range) (cm)	5.8 ± 3.9
UICC TNM stage	
Ⅰ	5
Ⅱ	41
Ⅲ	2
Edmonson-Steiner's grade	
Ⅰ	8
Ⅱ	13
Ⅲ	20
Ⅳ	5
Underlying liver disease	
Cirrhosis	22
Chronic hepatitis	26
AFP (ng/mL)	
< 11	12
11-400	16
⩾ 400	20

AFP: Alpha-fetoprotein.

5.8 ± 3.9 cm. Among the 46 patients with solitary tumors, a diameter of 3 cm or less was observed in 11 (23.9 %), between 3 cm and 5 cm in 15 (32.6%), and greater than 5 cm in 20 (43.5%). There were no tumors with positive lymph nodes or macroscopic vascular invasion by HCC. Of these HCCs, 8 were at grade Ⅰ, 13 at grade Ⅱ, 20 at grade Ⅲ and 5 at grade Ⅳ according to the Edmondson and Steiner's grade[25]. Microscopically, vascular and tumor capsular invasions were detected in 13 (27%) and 5 (10%) patients, respectively.

RT-PCR and sequencing of FHIT transcripts

Representative results of nested RT-PCR analysis are shown in Figure 1. Abnormal-sized bands representing aberrant RT-PCR products were detected in 11 (22.9%) of 48 surrounding non-tumorous liver tissues and 27 (56.3%) of the 48 HCC samples. Thus, the number of tumors with aberrant transcripts was greater than that of non-tumor tissues with aberrant transcripts (P = 0.002). Such abnormal-sized transcripts were seen at five different positions at 296, 365, 389, 413, and 458 bp (Figure 1). Interestingly, 13 cases displayed two or three aberrant transcripts. Sequence analysis of the aberrant transcripts revealed deletions of exons 3-6 (nt-164 to 249) in 17 cases, exons 4-7 (nt-110 to 279) in 7 cases, exons 4-8 (nt-110 to 348) in 6 cases, exons 5-7 (nt-17 to 279) in 6 cases, and exons 5-8 (nt-17 to 348) in 2 cases (Figure 2). Among 11 surrounding non-tumorous liver tissue samples with aberrant transcripts, 5 were chronic hepatitis, whereas in the remaining 6 cases, the parenchyma surrounding the tumor was cirrhosis.

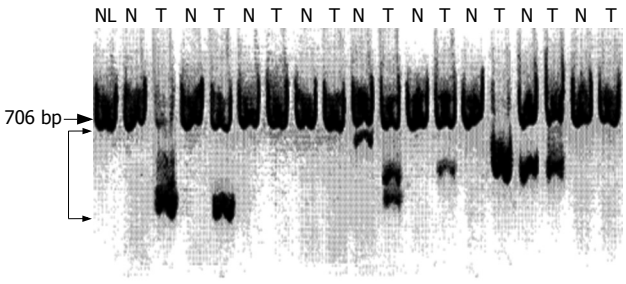

Figure 1 Analysis of mRNA expression of *FHIT* gene in matched surrounding non-cancerous and cancerous tissues by RT-PCR. Large arrow: Normal sized *FHIT* cDNA (706 bp); small arrows: Aberrant transcripts (458 bp, 413 bp, 389 bp, 365 bp, and 296 bp); NL: Normal liver tissue; T: Tumor tissue; N: Non-tumor liver tissue.

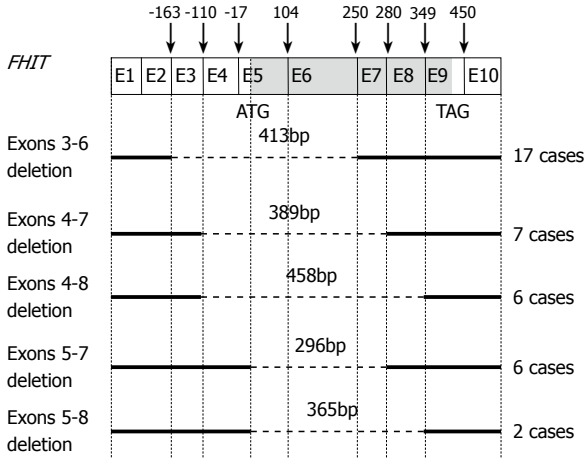

Figure 2 Schematic representation of aberrant *FHIT* transcripts in HCC tissues. Sequence analysis of aberrant transcripts reveals deletions of exons 3-6 (nt-164 to 249) in 17 cases, exons 4-7 (nt-110 to 279) in 7 cases, exons 4-8 (nt-110 to 348) in 6 cases, exons 5-7 (nt-17 to 279) in 6 cases, and exons 5-8 (nt-17 to 348) in 2 cases.

In total, 8 patients displayed aberrant transcripts in both tumor and surrounding non-tumor tissues, 5 of whom displayed the same abnormal patterns in the paired tumor and non-tumor samples; however, in the remaining 3 cases, the pattern was different between the paired samples. Whereas 19 cases had aberrant transcripts only in their tumor tissues, we also found 3 cases showing an aberrant transcript in their non-tumor tissues only. However, no point mutations of the *FHIT* gene were found in the region of the open reading frame. All normal-sized transcripts of 706 bp exhibited wild-type sequences. All of 21 tumor tissues without aberrant transcripts were presented normal sized transcripts. Normal-sized transcripts were also observed in 22 of the 27 HCCs with aberrant *FHIT* transcripts, but were barely present or completely absent in the remaining 5 cases. However, normal-sized bands were observed in all surrounding non-tumor tissues samples, regardless of the presence of aberrant transcripts. The presence of *FHIT* aberrations was not associated with clinical parameters, including age, sex, Child-Pugh classification, histological grade of tumor, presence of tumor capsule, AFP, tumor size, microscopic invasion, tumor recurrence, or survival (P > 0.05).

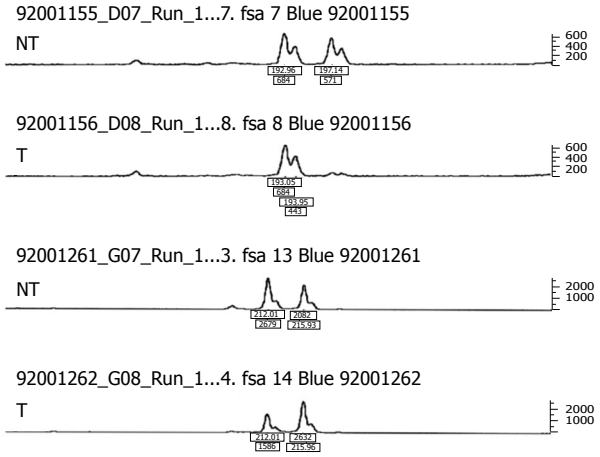

Figure 3 LOH of *FHIT* locus. An example of LOH in tumor (T) and matched non-tumorous (NT) DNA from patients with HCC. LOH is defined as if the signal of one allele is lost or reduced to at least 50% in tumor DNA, compared to the corresponding normal allele.

Loss of heterozygosity (LOH)

Among the 48 HCCs, 7 displayed LOH of D3S1300, D3S1313, or both markers. Specially, 4 of the 42 informative cases at D3S1300, and 3 out of the 29 cases informative at D3S1313, exhibited LOH. LOH involving both allelic markers was not observed. Examples are shown in Figure 3. In total, 6 of 30 cases with aberrant transcripts, and only 1 of 18 cases without aberrant transcripts showed LOH. Thus, the incidence of LOH appeared higher in cases with aberrant transcripts than cases without aberrant transcripts, although the data were not statistically significant ($P = 0.23$). Moreover, we observed a trend towards *FHIT* aberration in tumors with LOH, although again the difference was not statistically significant ($P = 0.23$). LOH of the *FHIT* gene was not associated with the clinical parameters examined.

Western blot analysis

Representative results of Western blot analysis are shown in Figure 4. Eight (16.7%) of 48 HCCs exhibited reduced or no Fhit expression, compared with normal liver tissues. Complete loss of Fhit was only identified in one of the above 8 cases. Reduced Fhit expression was observed in 4 among the 30 samples with aberrant transcripts. The remaining 4 cases with reduced Fhit did not show aberrant transcripts. Moreover, Fhit expression was not reduced in the 5 cases where normal transcripts were absent or barely detected. Reduced Fhit expression was observed in only one patient displaying LOH at *FHIT* with no aberrant transcripts. The remaining six cases with reduced Fhit expression did not display LOH at the *FHIT* locus. We observed no association between LOH and abnormal *FHIT* transcripts, abnormal *FHIT* transcripts and abnormal Fhit expression, or, LOH and abnormal Fhit expression. Moreover, the extents of Fhit were not associated with clinical parameters, such as age, sex, Child-Pugh classification, histological grade of tumor, presence of tumor capsule, AFP, tumor size, microscopic invasion, tumor recurrence, or survival ($P > 0.05$).

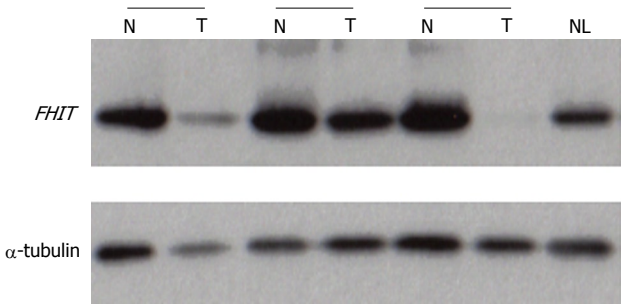

Figure 4 Representative results of Fhit expression by Western blot analysis. α-tubulin is used as the internal control to verify equal loading. The relative values of FHIT protein were normalized internally to α-tubulin signals. NL: Normal liver tissue; N: Non-cancerous tissue; T: Tumor tissue.

Table 2 *p53* mutation analysis in 48 patients with HCC

Mutation sites	*n*	Mutation sites	*n*
Exon 5		Exon 8	
Arg(CGC)158Leu(CTC)	1	Arg(CGT)273Cys(TGT)	1
Exon 6		Exon 7	
His(CAT)193Tyr(TAT)	1	Arg(TAC)236Cys(TGC)	1
Ser(AGT)215Arg(AGG)	1	Arg(TAC)236Asp(GAC)	1
Tyr(TAT)220Cys(TGT)	1	Met(ATG)246Leu(TTG)	1
		Met(ATG)246Val(GTG)	1

p53 mutation analysis

To investigate the association between *p53* mutations and genetic changes in the *FHIT* gene in the pathogenesis of HCC, direct sequencing analysis was performed. *p53* mutations were identified in tumors of 9 of the 48 patients (Table 2), but not in the surrounding liver tissues. However, none of the cases displayed G to T transversion at *p53* codon 249.

DISCUSSION

Common fragile sites are prone to genomic alterations in cancer, and the majority of cancers exhibit alterations at fragile sites[26]. The *FHIT* gene located at one of the most common fragile sites is altered in a variety of tumor cell lines, as well as, in premalignant and malignant lesions of primary tumors, in line with its role as a tumor suppressor gene whose loss or inactivation may contribute to cancer development or malignant progression[13,15-20]. Moreover, several studies have been reported results for chromosome 3p rearrangements, decreased or absent *FHIT* mRNA expression, intragenic deletions and absence of protein expression, in HCC cell lines and primary HCC[13,15-19,21-22].

Aberrant *FHIT* transcripts have been identified in 39%-70% of HCC cases[13,15-18]. These transcripts are generated by exon skipping, use of alternative 5' and 3' splice sites, and recognition of cryptic splice sites, resulting in insertions of intronic sequences. Consequently, the patterns of aberrant *FHIT* transcripts vary, but the majority of transcript splicing occurs within two large introns 4 and 5[15-18]. As the fusion functions coincide exactly with splice sites, aberrant transcripts may represent alternatively spliced products[9]. However, point

mutations of the *FHIT* gene are very rare[13,15-17]. In our study, all aberrant *FHIT* transcripts displayed deletion of exons 5 and 6. Additionally, no point mutations in the *FHIT* gene were identified in the region of the open reading frame. Aberrant transcripts of the *FHIT* have been identified in both HCC and surrounding non-cancerous tissues[15-18]. In our study, abnormal sized bands representing aberrant RT-PCR products were detected in 22.9% of the surrounding non-tumorous liver tissues and 56.3% of HCCs. These results suggested that aberrant *FHIT* transcripts had already accumulated even at the chronic liver diseases, in which persistent viral infection and sequential inflammation have occurred. As alterations of the *FHIT* gene in non-neoplastic tissues of smokers and ex-smokers are observed, *FHIT* alteration may be an early change during the preneoplastic phase of hepatocarcinogenesis[15-18]. Gramantieri *et al* reported that HCC cases with aberrant *FHIT* transcripts showed a significantly higher relapse rate and shorter recurrence time[14]. However, we observed no correlation between aberrant *FHIT* transcripts and clinicopathological parameters. Ohta *et al* detected full-length RT-PCR products in nearly all cases with aberrant transcripts[9]. Most of HCCs also exhibited aberrant and normal-sized transcripts[15-18]. Consistent with previous reports, our result showed that almost all cancerous and non-cancerous liver tissues exhibited normal-sized transcripts. In these cases, the normal transcripts have derived from admixed normal cells[9]. In our study, mRNA was extracted from tissue homogenates and, it was therefore not possible to assess whether or not the same cells produced normal and abnormal messengers. However, Chen *et al* reported that the normal products were observed in all tumor cell lines[17]. It has been suggested that these normal products are derived from neoplastic cells. Moreover, Schlott *et al* reported that aberrant transcripts did not differ between malignant, benign proliferating, and normal hepatocytes[16]. That is, formation of aberrant *FHIT* transcripts appears to be a common feature of benign, non-neoplastic hepatocytes. Alternative splicing definitely occurs in normal human tissues[27]. It is thus possible that the *FHIT* gene is simply located near to but is not the true target that drives a clonal selection process[17]. The *FHIT* gene, containing a common fragile region, FRA3B, is susceptible to the breakage caused by physical or chemical carcinogens. Similar effects may lead to a higher frequency of changes in the *FHIT* gene in chronically damaged liver tissues.

Several mechanisms are associated with dysfunction of the *FHIT* gene, the major being genomic deletion. These chromosomal deletions are frequently identified in HCC cell lines[13]. However, LOH at *FHIT* gene in human HCC is only occasionally detected[13,15,17]. Our study revealed that LOH of the *FHIT* gene was found in only 15% of HCCs. Although a great number of loci should be analyzed to further define any possible small deletions and rearrangements, these results so far suggest that LOH of *FHIT* gene is an uncommon in hepatocarcinogenesis. Allelic loss at *FHIT* is occasionally observed either in the presence or absence of aberrant transcripts[15]. In our study, LOH of *FHIT* gene was observed in 6 of 30 cases with aberrant transcripts and 1 of 18 cases without aberrant transcripts. However, no significant correlations were evident between the expression of aberrant transcripts and LOH of FHI. These finding imply that LOH alone does not completely suppress *FHIT* expression.

Several studies show that whereas all normal and non-cancerous liver tissues show a strong expression of Fhit, most HCC cell lines and primary HCC express reduced or no Fhit[13,19,21,22]. However, our study, reduced Fhit expression was observed in only 8 (17%) of 48 HCCs, and complete loss of Fhit in only one of these cases. Moreover, the extent of Fhit expression was not associated with aberrant transcripts or LOH presence. The data suggests that, in some cases, aberrant splice transcripts are actually transcribed. This hypothesis requires confirmation with a larger, more extended study. Inconsistent with our study, the incidence of Fhit expression was lower in HCC developed in patients chronically infected with HBV and exposed to chemical carcinogens, particularly in Qidong, China[13,19]. The exact reasons for the variation in Fhit expression are currently unclear, but may be dependent on different geographic and environmental factors. Hepatitis B virus infection is the most common etiologic factors for the development of HCC in South-East Asian regions, such as China and South Korea. Epidemiological and experimental studies disclose synergistic effects of aflatoxin B1 (AFB1) and HBV on hepatocarcinogenesis[28]. Environmental factors, including AFB1, may also contribute to the alteration of the *FHIT* gene. The concentration of AFB1 in the environment is high in China, but, low in South Korea[29]. AFB1 is strongly associated with a G to T transversion at codon 249 of the *p53* gene[28]. However, in our study, none of the cases displayed this G to T transversion. Loss or reduction of Fhit expression in HCC has been identified in association with altered *FHIT* transcripts, and LOH at the *FHIT* locus[13]. In our study, reduced Fhit expression was observed in only 4 of 30 cases with aberrant *FHIT* transcripts, and the remaining 4 cases displayed reduced Fhit expression did not present aberrant *FHIT* transcripts. Only one case with LOH at *FHIT* exhibited reduced Fhit expression. The six other cases with decreased Fhit expression did not show LOH at the *FHIT* locus. In contrast to previous studies, we observed no associations between Fhit expression and altered transcription or LOH. Loss of Fhit expression correlates with poor outcome in various cancers[14,30]. Zhao *et al* also reported that the expression of Fhit is inversely related to disease progression in HCC[19]. However, we found no correlation between Fhit expression and clinicopathological parameters. Although no interrelationship was evident between Fhit expression and LOH events, it is possible that other mutations, not investigated our study, are responsible for reduced or negative protein expression. Therefore, several genetic or epigenetic factors may potentially contribute to the loss of Fhit expression. One possibility is that inactivation

of the *FHIT* gene results from epigenetic mechanisms, such as hypermethylation of 5'-CpG islands in the promoter region[19,31]. In addition, abnormalities in post-transcriptional regulation may also abrogate expression of the *FHIT* gene. However, our results are preliminary and need to be confirmed in a larger study including more cases and an extended follow-up period.

In conclusion, our data indicate that abnormalities in *FHIT* gene transcripts occurred frequently in both cancerous and non-cancerous liver tissues. However, a normal-sized transcript without sequence abnormalities was almost always present. The Fhit is under-expressed only in a minor fraction of HCC tissues, while it was strongly expressed in non-cancerous liver tissues. Therefore, our study suggests that *FHIT* plays a role in relatively few HCC in lower AFB1 exposure area such as, South Korea. It is possible that such a finding was attributable to chance due to the relatively small numbers in our study. Thus, additional studies with more subjects are needed to confirm this finding.

COMMENTS

Background

The fragile histidine triad (*FHIT*) has been reported that this gene is a bona fide tumor suppressor gene. The *FHIT* gene and its product may be involved in the regulation of cell proliferative and apoptotic process. Down-regulation of *FHIT* inhibits apoptosis and *FHIT* has been shown to interact with a number of key proteins involved in cancer including *p53*

Research frontiers

Altered transcripts and allelic loss of the *FHIT* gene are frequently found in premalignant and malignant lesions of various tumors. Moreover, loss or reduction of *FHIT* protein (Fhit) expression has been found in most tumors including hepatocellular carcinoma (HCC).

Innovations and breakthroughs

Abnormalities of the *FHIT* gene transcripts occurred frequently in cancerous and non-cancerous liver tissues. However, a normal-sized transcript without sequence abnormalities was almost always present. Moreover, the Fhit was under-expressed only in a minor fraction of HCC tissues in lower AFB1 exposure area such as, South Korea, while it was strongly expressed in non-cancerous liver tissues. Therefore, none of the cases had a G to T transversion at *p53* codon 249.

Applications

FHIT behaves as a tumor suppressor gene whose loss or inactivation may contribute to HCC development or malignant progression in patients chronically infected with HBV and exposed to chemical carcinogens, particularly in areas from Qidong, China. In contrast, *FHIT* plays a role in relatively few HCC in lower AFB1 exposure area such as South Korea.

Peer review

This is an original study that has a large big amount of work. The authors concluded that *FHIT* plays a role in relatively few HCC's in South Korea. This is an interesting study, with sound methodology.

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