Alterations of the FHIT Gene in Human Hepatocellular Carcinoma

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ABSTRACT

FHIT (fragile histidine triad), a candidate tumor suppressor gene, encompasses FRA3B, a region with the highest fragility in the human genome, and is altered in a large number of human cancers, particularly those of epithelial cell origin and associated with known carcinogenic agents. Human hepatocellular carcinoma (HCC), a major cancer worldwide, is closely related to carcinogenic agents such as hepatitis B and C virus infections, dietary aflatoxin, alcohol consumption, and exposure to chemical carcinogens. To assess the extent and the nature of the FHIT gene alterations and their implications in the development of HCC, several cell lines and primary tumors were cytogenetically and molecularly examined. The FHIT gene is expressed in normal hepatic cells and is not expressed or is abnormally expressed in cultured tumor cells derived from HCC. Down-regulation of the FHIT gene was detected by Northern blot analysis in 9 of 14 cell lines. However, neither abnormal FHIT transcripts nor point mutations in DNA sequences of reverse transcription-PCR products (exons 2–9) were identified. Expression of FHIT protein was not detected by immunostaining in 5 of 10 primary tumors. Four cell lines showing mRNA down-regulation did not express FHIT protein as demonstrated by Western blot analysis. Allelic loss of intron 5 of the FHIT gene was detected in 10 of 34 informative samples from primary tumors. Structural alterations of chromosome 3p were identified in 8 of 13 HCC cell lines. Deletions or translocations involving region 3p14.2 were identified by fluorescence in situ hybridization with a YAC850A6 probe spanning the FHIT locus on chromosomes derived from cell lines with an abnormal FHIT gene expression. These combined results indicate that the FHIT gene is a frequent target and may be implicated in a subset of liver cancers.

INTRODUCTION

The FHIT gene, the first candidate tumor suppressor gene isolated in a critical region of recurrent deletions in major human cancers, has generated worldwide interest (1). Indeed, structural and functional alterations of the FHIT gene were identified in most common forms of cancer (2–4). There are several reasons for the interest in the FHIT gene; the most compelling one is its chromosomal location and tumor suppressor activity (1, 5). The FHIT gene is located on 3p14.2, a region of high fragility (FRA3B) and recombination as well as of suppressor activity (1, 5). The FHIT gene was detected by Northern blot analysis. Allelic loss of intron 5 of the FHIT gene was detected in 10 of 34 informative samples from primary tumors. Structural alterations of chromosome 3p were identified in 8 of 13 HCC cell lines. Deletions or translocations involving region 3p14.2 were identified by fluorescence in situ hybridization with a YAC850A6 probe spanning the FHIT locus on chromosomes derived from cell lines with an abnormal FHIT gene expression. These combined results indicate that the FHIT gene is a frequent target and may be implicated in a subset of liver cancers.

MATERIALS AND METHODS

Cell Lines and Tumor Tissue. All cell lines used in this study were cytogenetically analyzed in our laboratory and other laboratories by conventional G-banding, FISH, and comparative genomic hybridization. They were established from patients with HCC from different geographical locations and have an epithelioid HCC-like morphology and are tumorigenic in nude mice, and their viral profile is known (11). Normal human liver tissue was obtained from the National Cancer Institute, Surgery Branch, from individuals killed in accidents. The frozen HCC tissues were obtained from patients undergoing surgery in Qidong, China. Pathological diagnosis was determined using the H&E-stained specimens. Genomic DNA of the primary HCC and corresponding surrounding nontumor tissue were isolated by the standard phenol extraction procedure.

FISH. Chromosomes obtained from exponentially growing cultures were cohybridized with biotin- and digoxigenin-labeled whole chromosome or arm painting probes, the spectrum orange-/green-labeled probes (Life Technologies, Inc., Arlington, VA) and single-copy gene and YAC850A6 probes were used. The DNA probes were denatured for 5 min in 70% formamide/20× SSC. The probe mixture was applied on denatured chromosomes, and slides were incubated in a humidified chamber at 37°C for 16–24 h. Posthybridization washings were in 50% formamide/20× SSC. The probe mixture was applied on denatured chromosomes, and slides were incubated in a humidified chamber at 37°C for 16–24 h. Posthybridization washings were in 50% formamide/20× SSC. The final washings were performed in 0.1× SSC at 60°C or 2× SSC at 37°C for lower stringency. Detection of the hybridization signal, digital image acquisition, and analysis were carried out as described previously (12).

RNA Extraction and Northern Blot. RNA was extracted from HCC cell lines and normal liver tissue using TRIzol (Life Technologies, Inc., Grand Island, NY). Polyadenylated RNA was isolated by eluting total RNA from oligodeoxynthymidic acid column (Bio-Rad). One μg of mRNA for each sample was resolved on 1% agarose–4-morpholinepropanesulfonic acid/formaldehyde gel, transferred to a nylon membrane, and hybridized to a radiolabeled full-length FHIT cDNA probe of FHIT gene in Rapid-Hyb buffer (Amersham, Arlington Heights, IL). The blot was washed for 30 min at 62°C in 0.1× SSC/0.1% SDS, exposed to a phosphorimager plate, analyzed by software ImageQuant Version 3.3 (Molecular Dynamics, Sunnyvale, CA), and rehybridized to a β-actin cDNA probe. Immunohistochemical Staining. Formalin-fixed, paraffin-embedded primary tumor tissues were sectioned at 5 μm and transferred onto poly-t-lysine-coated slides and deparaffinized in two changes of xylene for 5 min each. Sections were hydrated by passing through a series of graded alcohols and steamed by 0.1 [scap]m sodium citrate buffer. FHIT antisera (provided by Dr. Kay Huebner, Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA) was used as the first antibody at a 1:1000 dilution. The following

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steps, including the addition of the second antibody and the development and staining, were performed according to the manufacturer’s instructions (Vector Laboratories, Inc., Burlingame, CA).

Western Blotting. Cells (1 x 10^7 cells/sample) were harvested and lysed in 50 μl of ice-cold lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 100 μM Na_3 VO_4, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin] for 1 h on ice. Cell lysates were collected by centrifugation at 14,000 rpm for 10 min, separated on a 4–20% SDS-polyacrylamide gel, and transferred electrophoretically to an Immobilon polyvinylidene difluoride membrane. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with rabbit anti-FHIT serum at a 1:1000 dilution and visualized following the instructions of an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, United Kingdom).

Microsatellite Allelic Loss. DNAs of human primary HCCs obtained from Qidong patients were used for the analysis of allelic loss within the FHIT gene.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Copy number of chromosome 3p</th>
<th>Copy number of YAC850A6</th>
<th>Alterations of chromosome 3p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sk-Hep-1</td>
<td>3</td>
<td>3</td>
<td>t(3;13)(p14;q13)</td>
</tr>
<tr>
<td>Hub-1</td>
<td>3</td>
<td>3</td>
<td>t(3;13)(p14;q13)</td>
</tr>
<tr>
<td>WRL</td>
<td>3</td>
<td>2</td>
<td>del(3)(p14;p14)</td>
</tr>
<tr>
<td>Chang</td>
<td>2</td>
<td>1</td>
<td>t(3;5)(p10;q10)</td>
</tr>
<tr>
<td>HepG2</td>
<td>2</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>2</td>
<td>2</td>
<td>t(3;7)(p10;7), t(3;7)(p10;7)</td>
</tr>
<tr>
<td>Focus</td>
<td>3</td>
<td>2</td>
<td>der(3)(t(3;7)(p10;7);del(3)(p14;p14), t(3;7)(p10;7)</td>
</tr>
<tr>
<td>Hep3B</td>
<td>4</td>
<td>2</td>
<td>der(3)(t(3;7)(p10;7);del(3)(p22;p10), t(2;3)(p22;p22), t(3;7)(p10;7)</td>
</tr>
</tbody>
</table>

Fig. 1. a, metaphase from the SK-Hep-1 cell line exhibiting an unbalanced translocation t(3;13) after cohybridization with a digoxigenin-labeled probe for chromosome 3p and a biotinylated YAC850A6 probe spanning FHIT gene. The breakpoint is near or within the YAC signal (yellow). b, metaphase from the Focus cell line after cohybridization with a digoxigenin-labeled probe for chromosome 3p and a biotinylated YAC850A6. Signal for YAC probe was detected on two copies of 3p. Three other regions of 3p did not exhibit signals, indicating interstitial deletions of the FHIT gene.

Table 1 Cytogenetic analysis of chromosome 3p in HCC cell lines

* Fifty metaphases cohybridized with chromosome 3p and YAC850A6 probes were examined, and the modal number of 3p and YAC signals is presented.
gene. Primer sequences were obtained from the Genome database [5'-AGCT-CACATTCTAGTCAGCCT-3' (forward primer) and 5'-GCCAATTCCCCAGATG-3' (reverse primer)]. The forward and reverse primers were labeled with fluorescein 6FAM and Hex (Perkin-Elmer, Norwalk, CT), respectively. The PCR reaction contained 0.1 μg of genomic DNA, 50 μM deoxynucleotide triphosphates, 1 μM fluorescein-labeled primer and unlabeled primer, 1.5 mM MgCl₂, 20 mM Tris (pH 8.5), 50 mM KCl, and 0.1 unit of Taq polymerase (Perkin-Elmer). In each sample, fluorescein 6FAM-labeled primer and Hex-labeled primer were used for tumor DNA and normal DNA, respectively. After an initial denaturation at 95°C for 3 min, a total of 25 cycles of PCR were

Fig. 2. mRNA expression of the FHIT gene in normal liver cells and HCC cell lines by Northern blot (A) and RT-PCR (B). A, Northern blot hybridization. The FHIT gene is expressed in normal liver cells but has low or undetectable expression in the majority of the cell lines. B, RT-PCR. Nested PCRs were performed. PCR products (620 bp) were not detected in Hub-7, HLF, 7703, Chang, Sk-Hep-1, Hub-1, Hep3B, and Focus cell lines. Point mutations were not detected in any DNA sequences of RT-PCR products.

Fig. 3. FHIT immunoreactivity in primary tumors. Normal liver cells showing positive FHIT immunostaining in the cytoplasm (A and B). A primary tumor showing FHIT-negative immunostaining, weak positive staining in fibroblast cells from cirrhotic nodes, and intense immunostaining of the surrounding normal liver cells (C and D).
integrity of all mRNAs was tested by successfully amplifying
observed in eight cell lines and in normal liver tissue (Fig. 2B). The
exon 2 to exon 9 of the
nested primer sets, a product of 620 bp was expected, which spans
and HCC cell lines as templates. After two rounds of PCR using the
by RT-PCR, using mRNAs extracted from human normal liver tissue
A very low level expression of the
approximately 1 kb was detected in normal liver tissue, but a decreased or
analysis on cells derived from HCC cell lines. A transcript of approxi-
ly 1 kb was detected in normal liver tissue, but a decreased or
Altering the short arm of chromosome 3 caused loss of the
FHIT gene copy number detected in eight cell lines (Table 1). Alter-
ations consisted of unbalanced translocations near or within the
FHIT gene and terminal and/or interstitial deletions of 3p (Table 1). In
cell lines 7703 and SK-Hep-1, the breakpoint in unbalanced translo-
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series of probes covering region 3p14.2–3p22 (12). In Fig. 1, FISH
with chromosome 3 and YAC8506A shows the breakpoint near the
YAC signal. Because this translocation is unbalanced, the possibility
exists that the rearrangement occurred within the FHIT gene. In other
cell lines, FOCUS, Huh-7, and WRL, interstitial deletions of the FHIT
gene are demonstrated by dual-color hybridization of YAC and 3p
probes (Table 1; Fig. 1).

The expression of FHIT mRNA was examined by Northern blot
analysis on cells derived from HCC cell lines. A transcript of approxi-
ately 1 kb was detected in normal liver tissue, but a decreased or
very low level expression of the FHIT gene was observed in at least
9 of 14 HCC cell lines (Fig. 2A). These cell lines were also exami-
ned by RT-PCR, using mRNAs extracted from human normal liver tissue
and HCC cell lines as templates. After two rounds of PCR using the
nested primer sets, a product of 620 bp was expected, which spans exon 2 to exon 9 of the FHIT gene. The expected PCR products were
observed in eight cell lines and in normal liver tissue (Fig. 2B). The integrity of all mRNAs was tested by successfully amplifying β-actin and other gene products. Point mutations were not detected by DNA sequencing of RT-PCR products of the FHIT gene.

FHIT protein expression was examined by immunohistochemical
staining in normal human liver tissue and 10 randomly selected
primary HCCs derived from patients in Qidong, China. Fifty percent
or more of tumors from patients in Qidong have a p53 gene mutation
at codon 249, which is considered to be related to aflatoxin exposure
and HBV infection (10). Strong staining of FHIT protein in the
cytoplasm was detected in normal liver cells, but 5 of 10 primary
tumors were negative for FHIT protein (Fig. 3). Due to the inherent
difficulties in accurately detecting the FHIT expression by immuno-
staining cells derived from HCC cell lines, five cell lines were
examined by Western blot. A M, 17,000 FHIT protein was detected in
Hep40 cells that exhibit a normal expression of FHIT mRNA and normal copies of chromosome 3p. Significantly, FHIT protein was not
detected in Huh-1, Huh-7, 7703, and Huh-6 cells, all of which exhib-
ted different degrees of FHIT mRNA down-regulation (Fig. 4).

Fluorescein-based microsatellite analysis of 35 primary tumors was
carried out to detect the allelic loss of locus D3S1300, located at the fifth
intron of the FHIT gene. In 34 informative cases, we observed two major
red bands and two major blue bands representing two alleles for tumor
cells and normal cells, respectively, in the gel profile. The information
generated from gel profile was translated into a figure profile by the
GeneScan 2.1 program. The stutter pattern (13) of microsatellite PCR
was observed for most cases. Of 34 tumors, 10 showed a pattern con-
sistent with allelic loss of microsatellite probes (Fig. 5).

RESULTS

Structural alterations of chromosome 3 were identified in 11 of 13
HCC cell lines analyzed by conventional G banding and FISH. To
detect alterations involving the FHIT locus, double-color FISH with
YAC8506A encompassing the FHIT gene and chromosome 3p painting
were used. The copy number of 3p and signals for the
YAC8506A probe were compared and presented as the modal number
(Table 1). Alterations of the short arm of chromosome 3 caused loss of the
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DISCUSSION

This study provides evidence for recurrent alterations of the FHIT
gene in HCC, a carcinogen-related form of cancer of epithelial cell
origin. Epidemiological data link HCC with exposure to several
carcinogens, chemical agents, and oncogenic viruses. Both chemical
carcinogens and oncogenic viruses are known to target preferentially
fragile sites (14–16).

The FHIT gene encompasses FRA3B, the most actively expressed
fragile site in humans (17), which is a region prone to breakage and
with an increased risk for carcinogen-induced damage. Compelling
evidence linking FHIT gene alterations to carcinogens has been pro-
vided from studies of lung cancer. In adenocarcinomas from smoking
subjects, loss of heterozygosity at the FHIT locus was significantly
higher due to the interaction of tobacco carcinogens and FRA3B (18).
Furthermore, smoking duration and asbestos exposure correlate with
FHIT exon deletion (19). Whereas in lung cancer, chromosome 3p
deletion is a hallmark diagnostic alteration (6), recurrent alterations of
the short arm of chromosome 3 in HCC nonrandomly involving
region 3p14.2 are a novel finding. Deviation from the modal number
of copies of FISH signals for YAC85060A was observed in four cell

Fig. 4. FHIT protein expression in HCC cell lines detected by Western blot analysis. A M, 17,000 FHIT protein was detected in Hep40 cells, but not in Huh-7, Huh-6, Huh-1, and 7703 cells.

Fig. 5. Allelic deletion of the fifth intron of the FHIT gene in primary tumors. Primers for D3S1300, located in the fifth intron of the FHIT gene, were used in the fluorescence labeling-based microsatellite analysis. Two major red bands and two major blue bands represent two alleles of tumor cells and normal cells, respectively. A pattern consistent with loss of heterozygosity was seen in four samples (93-01, 93-02, 92-27, and 93-03), in which the ratio of two alleles in tumor cells (red) is only half of that in normal cells (blue).
lines, suggesting nonclonal FHIT changes within these lines. The efficiency of hybridization may also account for the lack of signals in certain spreads. Cell population heterogeneity was documented in squamous cell carcinoma cell lines based on FHIT analysis by dual-color FISH (20). We present evidence for chromosome 3p rearrangements in 61% of the cell lines, a decreased or absent FHIT mRNA expression in 66% of the cell lines, and intragenic deletions and an absence of protein expression in 50% of primary tumors. These observations suggest that FHIT alterations play an important role in the development of a subset of HCC. As in other neoplasias in which FHIT DNA, RNA, and protein alterations have been analyzed (2), our data in HCC showed a correlation between these abnormalities. Several mechanisms may contribute to the dysfunction of the FHIT gene, with genomic deletion considered to be the major one. Allelic loss was found in only 30% of this series of primary tumors. However, a higher frequency might have been detected with the use of additional microsatellite markers within the FHIT locus. Down-regulation or absence of FHIT mRNA expression in a majority of HCC cell lines could be only partially attributed to chromosomal rearrangements within or near the FHIT locus or deletions of 3p resulting in loss of FHIT RNA copies. In four cell lines, PLC/PRF/5, HLF, WRL, and Huh-1, there was a discrepancy between the RT-PCR signal and cytogenetic data. Hypermethylation of the GC island in promoter region may also contribute to FHIT gene expression alterations (21). In addition, Western blot analysis of FHIT protein expression in several cell lines suggests that abnormality of posttranscriptional regulation could also abrogate the expression of the FHIT gene.

Alterations in the FHIT gene occur early in the tumor development, particularly in cancers associated with environmental carcinogens (22). Loss at the FHIT locus in nonneoplastic tissue of smokers and ex-smokers is indicative of early carcinogen-induced damage (23, 24). Similarly, HBV may cause damage to this locus early in the process of hepatocarcinogenesis. In two recent studies, aberrant FHIT transcripts were detected by RT-PCR in HCC as well as in nonmalignant hepatic tissue (25, 26). As in lung tissue exposed to carcinogens, aberrant FHIT transcripts in nonmalignant liver tissue may also reflect early damage caused by either HBV integration or carcinogens at FRA3B. The incidence of FHIT gene alterations should be elevated in HCC developed in patients chronically infected with HBVs or exposed to chemical carcinogens, particularly in areas from China and sub-Saharan Africa. Consistent with this notion is the absence of FHIT protein expression in 50% of primary tumors derived from Qidong, China. HBV exhibits striking similarities with retroviruses in terms of integration and requirements of RNA intermediate and reverse transcriptase for replication. A major limitation in evaluating the specificity of HBV integration is due to the ability of the integrated virus to trigger chromosomal rearrangements, hence the localization of viral sequences may not represent the initial site of integration (27). However, HBV integration in chromosomes 11 and 17 is frequent, and LOH of one or more markers on 11p was detected in several HCCs. Deletion of a DNA segment without its replication was considered as the most likely mechanism causing LOH (28). Detection of HBV sequences in FRA3B will allow the dissection of molecular alterations caused by viral integration in HCC with FHIT gene alterations. Ampere evidence for FHIT gene alterations in several major cancers including HCC underlines the importance of this gene in carcinogenesis.

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