Frequent Aberration of FHIT Gene Expression in Acute Leukemias


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ABSTRACT

We analyzed the mRNA expression of the FHIT gene by reverse transcription-PCR (RT-PCR) in 54 cases of acute lymphoblastic leukemia (ALL; 11 cases of T-cell ALL [T-ALL] and 43 cases of non-T-ALL) and 40 cases of acute myeloid leukemia (AML) (46% of the ALL cases and 55% of the AML cases). FHIT expression was abolished or markedly decreased. Only abnormal short bands were detected in 30% of the ALL cases and 5% of the AML cases. Eighteen of 19 abnormal transcripts had the same fusion of exons 2-7, and all lacked the starting codon in exon 5. No obvious normal-sized PCR products were detected in cases exhibiting abnormal transcripts. These findings suggest that the expression of functional FHIT protein was lost in the majority of ALL (76%) and AML (60%) cases. Differential quantitative PCR of exons 3-9 of the FHIT gene and RT-PCR of the PTPRG gene, which is centromeric to the FHIT gene, showed the presence of the target sequences. Fluorescence in situ hybridization analysis using probes covering exons 5 and 8 revealed no difference in the signal patterns between leukemia and normal cells, showing one or two signal doublets in more than 90% of nuclei, and indicated that gross segments of the FHIT gene were not homozogously deleted in these cases. A small number of transcripts with an aberrant fusion between exons 2 and 7 were detected by RT-PCR in the bone marrow cells from four healthy individuals. Granulocytes, lymphocytes, and monocytes in the bone marrow cells of a healthy individual contained transcripts with the same fusion. This unique fusion of exons 2 and 7 might be preferentially involved in therapy-related AML (8, 11) and AML (17).

INTRODUCTION

Cytogenetic abnormalities of the short arm of chromosome 3 (3p) have been reported in various tumors (1-6). In several hematological malignancies, del(3p) and the allelic loss of the short arm of chromosome 3 have been reported (7-11). For example, 3p abnormality has been reported to be involved in therapy-related AML (8, 11) and B-lymphoplastic leukemia (12). Among the chromosome 3 regions thought to harbor tumor suppressor genes, band 3p14.2 has recently been gathering interest. Region 3p14.2 contains an apyridinolytic enzyme, the FHIT gene in man had the enzyme property of a typical diadenosine 5',5'-pl,p3-triphosphate (Ap3A) hydrolase (16). Barnes et al. (17) reported that the FHIT gene in man had the enzyme property of a typical diadenosine 5',5'-P1,P3-triphosphate (Ap3A) hydrolase that changes Ap3A into ADP and AMP (17).

The gene spans approximately 1 Mb of DNA at chromosome band 3p14.2, which includes the familial renal cell carcinoma chromosome translocation breakpoint (between exons 3 and 4 of the FHIT gene), the most frequently expressed human constitutive chromosomal fragile site [FRA3B, which is telomeric to the t(3;8) translocation], and numerous homozygous deletions in various human cancers frequently involving exon 5 of the FHIT gene (15).

The number of reports on the alteration of the FHIT gene in human solid tumors has been increasing (18). Not only a loss of expression but alternatively fused transcripts have been observed in various tumors, e.g., stomach and esophageal cancers (15), SCLCs and NSCLCs (19), Merkel cell carcinoma (20), breast cancer (21), and deletions that are frequently observed in breast carcinomas and de novo acute leukemias (6, 14, 11).

Recently, the presumptive tumor suppressor gene FHIT (named after the fragile histidine triad), which is mapped on 3p14.2, was cloned and characterized using molecular genetics (15).

The FHIT gene, a member of the histidine triad (HIT) gene family, is a highly conserved gene homologous to a group of genes identified in eukaryocytes. This gene shows 69% similarity to a Schizosaccharomyces pombe enzyme, diadenosine 5',5'-P1,P4-tetraphosphatease asymmetrical hydrolase (16). Barnes et al. (17) reported that the FHIT gene in man had the enzyme property of a typical diadenosine 5',5'-P1,P3-triphosphate (Ap3A) hydrolase that changes Ap3A into ADP and AMP (17).

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In acute leukemias, we investigated the expression of this gene in ALL and AML samples using RT-PCR. Genomic analysis of several exons of the FHIT gene and exon 12 of the PTPRG gene, which is also located on band 3p14.2, and chromosomal analysis using FISH were performed to examine fragments of the gene encoding FHIT protein.

MATERIALS AND METHODS

Patients and Samples. Bone marrow samples were collected from patients after obtaining their informed consent. Fifty-four pediatric ALL cases including 40 newly diagnosed cases and 14 relapsed cases and 40 adult AML cases were included in this study. All of the samples contained more than 90% blasts. The phenotype of the ALL cases were as follows: (a) c-ALL (TdT, CD10, and CD19 positive), 32 cases; (b) B-ALL (TdT negative and CD19 and surface immunoglobulin positive), 2 cases; (c) pre-B-ALL (TdT, CD10, CD19, and cytoplasmic μ positive), 9 cases; and (d) T-ALL (TdT, CD5, and CD7

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4 The abbreviations used are: AML, acute myeloid leukemia; RT-PCR, reverse transcription–PCR; ALL, acute lymphoblastic leukemia; FISH, fluorescence in situ hybridization; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; T-ALL, T-cell ALL; c-ALL, common ALL; B-ALL, B-cell ALL; pre-B ALL, pre-B-cell ALL; TdT, terminal deoxynucleotidyltransferase.
positive), 11 cases. According to the French-American-British classification, 5, 12, 10, 7, 5, and 1 AML cases were classified as M1, M2, M3, M4, M5, and M6, respectively.

Cytogenetic studies were performed at diagnosis on 55 cases (individual karyotypes are not shown). Chromosomal abnormalities involving 3p14.2 were not detected.

The expression of the FHIT gene in normal bone blood cells was analyzed using bone marrow samples from four healthy individuals after obtaining their informed consent.

DNA and RNA Extraction and PCR Analysis. Genomic DNA was extracted from the bone marrow samples using a standard procedure (25). RT-PCR, including RNA extraction and cDNA synthesis, was performed as described previously (26).

Nested PCR was carried out to detect the message of the FHIT and PTPRG genes. For the FHIT gene, outer primers FH1107F and FH1033R were used for the first PCR, and inner primers FH129F and FH984R were used for the second PCR. For the PTPRG gene, outer primers PT1494F and PT2176R were used for the first PCR, and inner primers PT1528F and PT2155R were used for the second PCR. These primers were based on the full-length FHIT cDNA sequence (Ref. 15; GenBank accession number U09247) and the PTPRG cDNA sequence (Ref. 27; GenBank accession number L09247) and were adopted from the previous report by Panagopoulos et al. (22). The outer primers were located in exon 1 and 10, and the inner primers were located in exons 2 and 10. For the first PCR, we used a 50-μl reaction solution containing 0.25 μM each deoxynucleotide triphosphate, 2 mM MgCl2, 1 unit of Taq polymerase (Takara Shuzo, Shiga, Japan), 0.8 μM each of the outer primers, 10% (v/v) DMSO, and 1 μl of cDNA. After denaturing the mixture at 94°C for 5 min, 35 cycles were performed, consisting of 30 s at 53°C, 60 s at 72°C, and 30 s at 94°C, followed by a final elongation for 10 min at 72°C. One μl of the first PCR product was amplified in 50 μl of the second PCR mixture under the same conditions as those for the first PCR, except that the inner primers, 1.5 mM MgCl2, an annealing temperature of 56°C were used, and no DMSO was used. The inner primers for the FHIT gene amplified 21 bp of exon 1, exons 2–9, and 172 bp of exon 10. Ten μl of the PCR products were analyzed using 1.5% agarose gels stained with ethidium bromide.

To determine the homozygous deletion of the FHIT exons, the ability to amplify each exon of the FHIT gene was compared with the ability to amplify a region of the human β-globin gene as described previously (28). Genomic DNA fragments were amplified using primers of the β-globin gene and primers iex3F, iex3R, iex4F, iex4R, X2F, G1, iex6F, iex6R, iex7F, iex7R, iex8F, iex8R, iex9F, and iex9R. These primer pairs were used to amplify genomic fragments containing exons 3, 4, 5, 6, 7, 8, and 9 of the FHIT gene, respectively (15, 22, 29). Primers KM29 and GH21, which were specific for the β-globin gene, were adopted from the previous report (30). The annealing temperature was 56°C, and there were 25 reaction cycles. Ten μl of the PCR products were analyzed by electrophoresis through 1.5% agarose gels stained with ethidium bromide. The peak areas of the PCR products were quantified using a densitograph (ATTO, Tokyo, Japan). The dosages of the target gene (FHIT) were calculated in the FHIT/β-globin ratio and compared with that of the normal samples.

Sorting of Marrow Cells. Bone marrow cells were washed twice with Ca2+- and Mg2+-free PBS (PBS') containing 2% FCS and then passed through a stainless steel mesh. Cells were fractionated on a FACStar Plus (Becton Dickinson). Sorting gates were established for intermediate forward scatter and low side scatter. We sorted the monocytic, lymphocytic, and granulocytic cells according to the characters based on side scatter and forward scatter. The recovered cells were then washed twice with PBS', and the extraction of RNA, cDNA synthesis, and RT-PCR were performed thereafter. A small number of cells from each fraction were stained with May-Gruenwald/Giemsa solution. We confirmed that more than 80% of the cells were classified into a specific lineage.

FISH Analysis. FISH analysis of interphase chromosomes was performed in 4 normal samples and 12 leukemic samples using probes C76 (covering exon 5 and flanking regions) and CO59#9 (spanning exon 8; Ref. 31) labeled by nick translation with digoxigenin-11-dUTP and biotin-16-dUTP, respectively. The probes were kindly provided by Drs. C. M. Croce and K. Huebner (Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA). The 12 leukemic samples consisted of 4 samples expressing normal PCR products (2 AMLs and 2 ALLs), 4 samples showing a loss of expression (2 AMLs and 2 ALLs), and 4 samples with abnormal PCR products (2 c-ALLs and 2 T-ALLs).

The FISH probe nick translation, chromosomal in situ suppression hybridization, and detection were carried out as described previously (32). Approximately 200 nuclei were counted for each sample. Only subpopulations representing at least 10% of the total number of the nuclei were considered in the evaluation.

Sequence Analysis. Abnormal PCR products were size-fractionated on 1.5% agarose gels, purified using QIAEX II (Qiagen, Hilden, Germany), cloned into pCRII plasmid vector (Invitrogen, San Diego, CA), and sequenced using a T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden). Alternatively, a DNA sequencing system (model 373A; Applied Biosystems, Foster City, CA) was used to sequence the cloned fragments.

RESULTS

The Expression of the FHIT Gene Was Frequently Abnormal in Acute Leukemias. To investigate the potential abnormality of FHIT gene transcription in acute leukemias, we used RT-PCR to examine the transcription patterns of the FHIT gene in 54 ALL and 40 AML samples.

By RT-PCR using the nested primers spanning exon 1 and exon 10, the wild-type PCR product, which is 855 bp, was detected in 13 (24%) ALL and 16 (40%) AML samples. The expression was absent or markedly decreased in the other 25 (46%) ALL and 22 (55%) AML samples. Shorter aberrant PCR products, from ~120 to ~520 bp in length, were observed in 16 (30%) ALL samples and 2 (5%) AML samples. Fig. 1 shows the PCR results of these cases. T-ALL (6 of 11 samples) had a significantly high incidence of abnormal message when compared with that of non-T-ALL (10 of 43 samples) using the χ2 test (P < 0.05).

Interestingly, no concomitant normal transcripts were clearly observed in the cases exhibiting abnormal transcripts. We also performed RT-PCR of the PTPRG gene, which is mapped centromeric to FHIT.
the FHIT gene. All of the cases exhibited normal transcripts of the PTPRG gene (Fig. 1).

**Aberrant Transcripts of the FHIT Gene Were Derived from Alternative Fusion of Exons.** Sequence analysis of the normal-sized transcripts from four ALL and three AML cases did not reveal any mutations. The sequences of 19 unusual-sized transcripts from 16 ALL and 2 AML cases are summarized in Table 1. Most notably, 13 samples (L24, L29, L37, L62, L80, L101, L114, L128, L133, L139, L169, M32, and M37) had the same 431-bp sequences, which lacked exons 3–6, leading to a fusion of exon 2 with exon 7 (exon 2-exon 7 fusion). We also found a common FHIT transcript with an alternative splicing that contained an 11-bp deletion at the beginning of exon 10 of the gene, as reported previously (Refs. 19 and 23; Fig. 2). The loss of exons 4–9, leading to a fusion of exon 3 with exon 10, was observed in the L82 sample.

Because all abnormal transcripts lacked exon 5, which contains the in-frame ATG (methionine) start codons of the FHIT gene open reading frame, it seems unlikely that they code for any functional protein. Together with the absence of FHIT expression, the FHIT gene was abolished in 41 (76%) ALL and 24 (60%) AML cases.

**Aberrant Transcripts Were Observed in Normal Bone Marrow Cells.** To analyze the expression of the FHIT gene in normal hematopoietic cells, we amplified the mRNA of bone marrow cells from four healthy individuals. In all cases, electrophoresis of the PCR products revealed a definite band of normal size, and no additional band could be observed on the agarose gel stained with ethidium bromide. We then cloned the whole PCR products of four individuals into the pCRII vectors. We screened the inserts by picking up a small amount of colonies with subsequent amplification. We selected five colonies with a short insert by PCR and purified the DNA by mini-prep. Sequence analysis revealed that all of them had exon 2-exon 7 fusion. We then fractionated the nucleated cells of bone marrow from a healthy individual into granulocytes, lymphocytes, and monocytes by fluorescence-activated cell sorting. Only the amplicon with a normal band size was observed in each fraction. After cloning the whole PCR product from each fraction into the vector, we selected two colonies with short inserts, respectively. The subsequent sequence analysis revealed that all of them had the transcript with the exon 2-exon 7 fusion, although cross contamination between fractionated cells could not be completely excluded.

**Alteration of the FHIT Gene Might Not Be Caused by Intrageneric Homozygous Deletion.** We examined the genomic DNAs and chromosomes for the presence of gross alterations in the FHIT gene that might be responsible for the identified alterations in expression using genomic PCR, RT-PCR, and FISH.

Differential PCR of exons 3, 4, 5, 6, 7, 8, and 9 was conducted to examine whether any deletions are confined within the FHIT locus (Fig. 3). For all exons, the expected PCR products were observed in all of the samples, and the FHIT:β-globin ratios showed no statistical difference from those of normal samples (P > 0.05; data not shown).

In addition to these examinations, we analyzed the existence of exons 5 and 8 of the FHIT gene on interphase nuclei by FISH using the probes C76 (covering exon 5) and C05#9 (covering exon 8). The cosmids C76 was detected with rhodamine-conjugated antidigoxygenin, which gives a red signal, and C05#9 with fluorescein-tagged avidin, which gives a green signal. In all samples, whether from normal cells or leukemias with normal or aberrant FHIT expression, the fraction of the nuclei showing one or two signal doublets did not differ significantly (Table 2).

These results indicate that there is little or no evidence that gross loss of genetic materials of the FHIT gene occurred in cases of either loss of expression or aberrant fusion transcripts.

**DISCUSSION**

In this study, we found frequent aberration of the FHIT gene expression in acute leukemias. Loss of gene expression was observed in 46% of the ALL cases and 55% of the AML cases. Abnormal short
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that lacked three or more exons (20). In breast cancer cell lines and primary tumors, about 20–30% of the samples exhibited altered transcripts (21, 34). Loss of expression was observed in another 10% of the primary tumors (21). In head and neck squamous cell carcinomas, 85% of the cell lines showed alterations of at least one allele (31). In hematological malignancies, 3p deletions have been reported in 2.9% of AML cases and 1.5% of ALL cases (11), and homozygous deletions have been found at locus D3S1284 in 3 of 29 chronic lymphocytic leukemia cases (35).

Aberration of the FHIT gene expression in various leukemias was first reported by Sugimoto et al. (36). They examined 40 leukemia samples including 16 AMLs, 5 ALLs, and 9 chronic myeloid leukemias (CMLs) in the chronic phase; 4 CMLs in the acute phase; and 6 chronic lymphocytic leukemia cases. They found a short PCR product in 36 of 40 leukemic samples (36). Direct sequencing revealed that all of them had abnormal transcripts lacking exons 3–6, as in the present study. The most notable difference was that a normal PCR product was observed in addition to the abnormal one in the majority of their cases. In addition, they did not find any loss of gene expression in the cases. Although the numbers of cases examined were rather small in both studies, these discrepancies might be explained by the content of leukemic blasts in the samples. We selected samples that contained at least 90% leukemic cells to minimize the contamination of the expression from the normal cells. In contrast, they enrolled samples that

bands were detected in 30% of the ALL cases and 5% of the AML cases and were the only transcripts of the FHIT gene. The sequence analyses of the aberrant transcripts revealed that all of them lacked exons 4 to 6 as a minimum region that includes the starting codon in exon 5. It is of interest that 18 of the 19 samples had the same exon 2-exon 7 fusion, which was an authentic splicing variant of FHIT. This was not observed in the various solid tumors reported by others. In addition, no normal-sized PCR products were detected in cases exhibiting abnormal transcripts. Therefore, all of the cases with either aberrant transcripts or the absence of transcripts might lack the FHIT protein. Because the aberrant transcript lacked exon 5, which contains the starting codon and the expression from the other allele, the gene could not be transcribed. Together with the lack of the expression, the majority of ALL (76%) and AML (60%) cases seemed to lack the functional FHIT protein.

Various solid tumors have been reported to show alteration of the FHIT gene, either as a loss of expression or as short transcripts lacking two or more exons (18). SCLCs (80%) and NSCLCs (40%) showed abnormal transcripts, and 76% of the samples exhibited the loss of FHIT alleles (19). Yanagisawa et al. (33) observed a lack of detectable expression or the exclusive expression of aberrantly spliced transcripts in 29% of the NSCLCs but in none of the SCLCs. Fifty-seven percent of Merkel cell carcinomas displayed abnormal products

Fig. 2. Sequence analyses of the abnormal transcripts of the FHIT gene from ALL samples. A shows an aberrant fusion between exons 2 and 7 (case L24). B and C show the alternative splicing between exons 9 and 10. The initial 11 bases underlined in the normal individual (B) are lacking in an ALL case (C, case L72).

Fig. 3. Representative results of quantitative differential PCR using primers specific for exons 3–9 of the FHIT gene. The β-globin gene was amplified as a reference gene. Lane 1, a healthy donor; Lanes 2–12, ALL cases (the same cases as indicated in Fig. 1). The ability to amplify each exon of the FHIT gene was not markedly diminished in any case, suggesting no homozygous loss of the exons. The size markers are pBR322 MsiI digests.
contained up to 40% of normal cells, and this might also explain the low incidence of loss of expression in their study.

In solid tumors, the major mechanism of loss of the FHIT transcripts has been assumed to be a homozygous loss of this gene. Some deletions might originate through breaks in fragile sites involving introns 4 and 5 (37). In our series of acute leukemia cases having aberrant expressions or loss of expression, genomic analysis of exons 3, 4, 5, 6, 7, 8, and 9 of the FHIT gene as well as FISH analysis of exons 5 and 8 was performed to examine the FHIT gene material. The data suggested that the gross segments of the FHIT gene were not homozygously deleted in these cases, although any small or noncontiguous homozygous deletion that was restricted within an intron could not be excluded. Mechanisms other than homozygous deletion, such as mutations of the promoter region or the dysfunction of regulatory proteins, might be involved in the repression of expression of the FHIT gene in acute leukemias.

The short transcripts were presumably derived from alternative splicing, because they were also observed in the normal samples, and because none of the cases having abnormal transcripts carried intronic homozygous deletions according to either the differential PCR or FISH analysis.

The splicing patterns of abnormal transcripts might correlate with the type of tumor or tissue. In previous reports on the FHIT aberration of solid tumors, the fusion of exon 3 and exon 7, 8, or 9 and the fusion of exon 4 and exon 8 or 9 were seen in NSCLC, and the fusion of exons 3 and 7 and the fusion of exons 3 and 9 were preferentially seen in SCLC and Merkel cell carcinomas (15, 20). In gastrointestinal cancers, the fusion of exon 4 and exon 6, 7, 8, or 9 was frequently observed (15). Hayashi et al. (34) reported that the abnormal transcripts from breast cancer cell lines were categorized into two types: (a) the fusion of exons 4 and 8 (type I); and (b) the fusion of exons 4 and 9 (type II). In the present study, the ALL and AML cases had aberrant transcripts preferentially lacking exons 3–6, leading to the exon 2-exon 7 fusion in the present study.

Recently, Panagopoulos et al. (38) reported that variable splicing transcripts of the FHIT gene are also present in nonneoplastic normal tissues. They also found the exon 2-exon 7 fusion transcript in normal peripheral blood lymphocytes. To examine the expression of the FHIT gene in normal hematopoietic cells, we obtained bone marrow cells from a healthy individual and sorted them by fluorescence-activated cell sorting into the granulocytic, monocytic, and lymphocytic lineage. We obtained bone marrow cells from a healthy individual and sorted them by fluorescence-activated cell sorting into the granulocytic, monocytic, and lymphocytic lineage. Gene in normal hematopoietic cells, we obtained bone marrow cells from a healthy individual and sorted them by fluorescence-activated cell sorting into the granulocytic, monocytic, and lymphocytic lineage.

Table 2 FISH analysis in cases with normal and abnormal FHIT transcripts

<table>
<thead>
<tr>
<th>Origin of the sample</th>
<th>Expression pattern of the FHIT gene</th>
<th>No. of cases examined</th>
<th>Subpopulation [mean ± 2 SD (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy individuals</td>
<td>Normal transcripts</td>
<td>4</td>
<td>71.5 ± 10.6 12.3 ± 7.0 10.0 ± 2.3</td>
</tr>
<tr>
<td>Leukemia cases</td>
<td>Normal transcripts</td>
<td>4</td>
<td>68.0 ± 11.4 12.0 ± 8.2 10.3 ± 8.0</td>
</tr>
<tr>
<td>Leukemia cases</td>
<td>Absent</td>
<td>4</td>
<td>62.0 ± 10.5 13.7 ± 5.8 15.0 ± 6.2</td>
</tr>
<tr>
<td>Leukemia cases</td>
<td>Absent</td>
<td>4</td>
<td>17.5 ± 7.0 12.5 ± 4.2</td>
</tr>
</tbody>
</table>

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