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# Hypermethylation of the 5′ CpG Island of the *FHIT* Gene Is Associated with Hyperdiploid and Translocation-Negative Subtypes of Pediatric Leukemia

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## ABSTRACT

**The human *FHIT* (fragile histidine triad) gene is a putative tumor suppressor gene located at chromosome region 3p14.2. Previous studies have shown that loss of heterozygosity, homozygous deletions, and abnormal expression of the *FHIT* gene are involved in several types of human malignancies. A CpG island is present in the 5′ promoter region of the *FHIT* gene, and methylation in this region correlates with loss of *FHIT* expression. To test whether aberrant methylation of the *FHIT* gene may play a role in pediatric leukemia, we assessed the *FHIT* methylation status of 10 leukemia cell lines and 190 incident population-based cases of childhood acute lymphocytic and myeloid leukemias using methylation-specific PCR. Conventional and fluorescence *in situ* hybridization cytogenetic data were also collected to examine aneuploidy, t(12, 21), and other chromosomal rearrangements. Four of 10 leukemia cell lines (40%) and 52 of 190 (27.4%) bone marrows from childhood leukemia patients demonstrated hypermethylation of the promoter region of *FHIT*. Gene expression analyses and 5-aza-2′-deoxycytidine treatment showed that promoter hypermethylation correlated with *FHIT* inactivation. Among primary leukemias, hypermethylation of *FHIT* was strongly correlated with acute lymphoblastic leukemia (ALL) histology ($P = 0.008$), high hyperdiploid ($P < 0.0001$), and translocation-negative ($P < 0.0001$) categories. Hyperdiploid B-cell ALLs were 23-fold more likely to be *FHIT* methylated compared with B-cell ALL harboring *TEL-AML* translocations. *FHIT* methylation was associated with high WBC counts at diagnosis, a known prognostic indicator. These results suggest that hypermethylation of the promoter region CpG island of the *FHIT* gene is a common event and may play an important role in the etiology and pathophysiology of specific cytogenetic subtypes of childhood ALL.**

## INTRODUCTION

Pediatric leukemias are a heterogeneous group of malignancies. Major cytogenetic subgroups exist that are characterized by structural chromosomal abnormalities leading to the synthesis of oncogenic fusion proteins and other subgroups with nonrandom gains or losses in chromosome number (1, 2). Some of these cytogenetic subtypes are mutually exclusive, indicating distinct pathogenetic pathways. In addition, a significant fraction of acute leukemias in children display apparently diploid karyotypes. Among the mechanisms considered in the pathogenesis of leukemias without structural alterations are aberrant methylation and associated transcriptional silencing of putative tumor suppressor genes. Several targets of epigenetic silencing have been identified in leukemia (3–13). Here we have focused on the fragile histidine triad (*FHIT*) locus because multiple reports indicate aberrant expression of the *FHIT* gene in leukemic cells (14–21). The human *FHIT* gene is a member of the histidine triad gene family (22, 23), the function of which remains unknown. *FHIT* knockout mice have an increased susceptibility to spontaneous tumors as well as being exquisitely sensitive to carcinogens (24, 25), and transfection of *FHIT* into tumorigenic cell lines inhibits tumorigenicity in mice (26). All of these data are compatible with the idea that *FHIT* is a tumor suppressor gene. The FHIT protein may function in the metabolism of polyphosphorylated diadenosine (*e.g.*, Ap3A) substrates that can be induced in hematopoietic cells by cytokines and which may participate in intracellular signaling pathways and mediate antiviral mechanisms (27–30). Other observations indicate that the activation of caspase-8 was correlated with *FHIT*-mediated apoptosis, which suggests that *FHIT* might exert a proapoptotic function through a caspase-mediated pathway (31, 32).

The promoter region around exon 1 of the *FHIT* gene contains a CpG island that has been shown to be hypermethylated in esophageal, lung, breast, prostate, bladder, cervical, and oral cancers (33–38). Aberrant methylation of the promoter region of the *FHIT* gene is strongly associated with gene inactivation as indicated by Northern blot, reverse transcription (RT)-PCR, and immunostaining analyses (33, 34). Hypermethylated cells can be demethylated and induced to re-express the *FHIT* gene products after treatment with 5-aza-2′-deoxycytidine. Also of interest is the finding that *FHIT* alterations, including methylation, may be associated with environmental exposures (34). Links between *FHIT* methylation and environmental exposures could make it a useful marker for epidemiological research exploring possible causal pathways in pediatric leukemia. Leukemia cases examined in this study are participants in a population-based etiological study of childhood leukemia in Northern California. We measured DNA methylation in the promoter region of the *FHIT* gene in 190 consecutive cases of childhood leukemia and assessed the relationship between *FHIT* hypermethylation and clinicopathological and cytogenetic parameters.

## MATERIALS AND METHODS

**Cell Lines and DNA Isolation.** Ten human leukemia cell lines (Molt-4, KG1A, Jurkat, RCH, Reh, Blin, NALM, 697, K562, and HL-60) from the American Type Culture Collection were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and were grown at 37°C in 5% $CO_2$. DNA was isolated from cultured cells using QIAamp DNA Mini kit (Qiagen Inc.) and quantified by fluorometery.

**Study Population.** Included in the *FHIT* methylation analysis were a total of 190 incident cases of childhood leukemia, patients who were enrolled in the Northern California Childhood Leukemia Study (NCCLS) and our studies used cryopreserved pretreatment bone marrow aspirates obtained from the clinical center that first diagnosed the case. A detailed description of this population-based study design can be found elsewhere (39–41). These patients were diagnosed between August 1995 and July 2000 in nine major clinical centers in the San Francisco Bay Area and Central Valley of California and were representative of the large case population (>88% of all newly diagnosed cases were included in this study). One hundred and fifty-six of the patients were diagnosed with acute lymphoblastic leukemia (ALL), 32 with acute myeloid leukemia (AML), and 2 with chronic myeloid leukemia. One hundred and four (54.7%) of the patients were male, and 86 (45.3%) were female. The

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Table 1 *Clinical and demographic characteristics of leukemia patients*

| Characteristics | *N* (number of patients) |
|---|---|
| Total | 190 |
| Gender | |
| Male | 104 |
| Female | 86 |
| Age (years) | |
| <2 | 19 |
| 2–5 | 95 |
| 6–14 | 76 |
| Subtype | |
| ALL[a] | 156 |
| AML | 32 |
| CML | 2 |

[a] ALL, acute lyphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia.

age of the patients ranged from 0.2 to 14.9 years, and the mean and median ages were 6.1 and 5.0 years, respectively. Of all of the cases, 48.9% were non-Hispanic White, 33.0% were Hispanic, 5.1% were African American, 4.0% were Asian American, and 9.1% belonged to other racial/ethnic groups. The clinical characteristics of leukemia patients are given in Table 1. Immunophenotype, cell counts at diagnosis, and diagnostic cytogenetics were abstracted from patient records and merged with results from the epidemiological, fluorescence *in situ* hybridization (FISH), and methylation analyses. More detailed information about distribution of the immunophenotypes and cytogenetic abnormalities as well as comparison to other population-based studies can be found in Table 2. Data on *p15INK4b* methylation were also available for these patients.

**Sample Processing of Bone Marrow and Bisulfite Modification of DNA.** Because bone marrow samples are very limited and there is DNA loss in any extraction procedure, a "One Step" sample processing and DNA modification technique was developed in this study to maximize DNA yield from limited samples. Briefly, to a microcentrifuge tube containing 13 μl of PBS, 5 μl of bone marrow aspirate and 1 μl of proteinase K were added. After mixing briefly, 17 μl of buffer AL (both proteinase K and AL buffer were from QIAamp DNA Mini kit; Qiagen Inc. Valencia, CA) were added and mixed, and samples were incubated at 56°C for 1 h. Bisulfite modification was as described previously (42, 43). To the above reaction tube, 4 μl of 3.0 M NaOH, freshly prepared, was added, and the DNA sample was denatured at 37°C for 15 min. The sample was then treated with 416 μl of 3.6 M sodium bisulfite solutions (pH 5.0) and 24 μl of 10 mM hydroquinone, both freshly prepared, and samples were incubated under 2 drops of mineral oil at 55°C for 16 h. For each set of modification, DNA extracted from peripheral blood of healthy individuals was included as control for the unmethylated version, and DNA from healthy individuals treated with Sss I methylase (New England BioLabs, Inc., Beverly, MA) was also included as positive methylation control.

Bisulfite-modified DNA samples were then purified using the Wizard DNA Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions and eluted twice in a total of 60 μl of 10 mM Tris (pH 7.6) preheated to 70°C. Freshly prepared NaOH, to a final concentration of 0.3 M, was added, and the sample was incubated at 37°C for 15 min. The solution was neutralized by addition of ammonium acetate (pH 7.0) to 3 M, and the DNA was ethanol precipitated, dried, and resuspended in 30 μl of 10 mM Tris buffer.

**Methylation Status by Methylation-Specific PCR (MS-PCR).** Detection of methylated CpG dinucleotides within the promoter region CpG island of the *FHIT* gene was carried out using MS-PCR (44), and primers (Qiagen Operon, Alameda, CA) for both methylated and unmethylated CpG sites were as described previously (34). Methylation detected with this assay was demonstrated previously to be significantly associated with loss of gene expression in lung cancer cell lines and primary lung and breast tumors (34); methylated CpG site, forward 5′-TTGGGGCGCGGGTTTGGGTTTTTACGC-3′ and reverse 5′-CGTAAACGACGCCGACCCCACTA-3′, unmethylated CpG site, forward 5′-TTGGGGTGTGGGTTTGGGTTTTTATG-3′, and reverse 5′-CATAAACAACACCAACCCCACTA-3′; GenBank accession no. is U76263, with an amplicon of 189–262 bp relative to transcription start site. The PCR mixture contained 10× PCR buffer (Applied Biosystems), $MgCl_2$ (1.5 mM final), deoxynucleotide triphosphates (0.2 mM each), primers (0.4 μM each), 1 unit AMPliTAQ DNA polymerase treated with TaqStart Antibody (CLONTECH Laboratories, Inc., Palo Alto, CA), and 2 μl of modified bone marrow DNA templates in a total volume of 25 μl. The PCR reactions were cycled in a GeneAmp 9600 thermal cycler (Applied Biosystems) under the following conditions: preheat at 94°C for 3 min., 94°C for 30 s, 65°C for 30 s, 72°C for 30 s for 38 times, and a final extension at 72°C for 7 min. For each PCR set, DNA samples from peripheral blood of normal blood donors treated with CpG methylase (M.Sss I; New England BioLabs) and bisulfite were included as positive controls, and no template reaction was included as negative control. In addition, we repeated MS-PCR assays on 30% of primary tumor specimens and found no discordant results among replicates. Aliquots (12 μl) of MS-PCR products were analyzed on 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination. Results were recorded with a digital image system.

**Bisulfite Genomic Sequencing.** To confirm the efficiency of the bisulfite modification and the specificity of methylation-specific PCR, direct sequencing of the PCR products was carried out as described previously (43). Briefly, PCR products were ligated into the PCR 2.1-TOPO plasmid vector using TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Purified plasmid DNA containing *FHIT* gene amplicon was sequenced in both directions using an ABI 377 automated sequencer with standard M13 primers. Two control samples from healthy individuals, two leukemia cell lines, and four *FHIT*-methylated bone marrow samples were directly sequenced.

***FHIT* Expression by RT-PCR and Western Blot.** cDNAs were synthesized from 3 μg of total RNA extracted from leukemia cell lines and representative primary leukemia bone marrows using Qiagen RNeasy Mini kits. The cDNA concentration was then normalized in series of PCRs with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers [5′-TCGTGGAAGGACTCATGACC-3′ (sense) and 5′-GGGATGATGTTCTGGAGAGC-3′ (antisense); 115 bp transcript fragment] by carefully diluting cDNA samples until PCR products of different samples were similar to each other in band

Table 2 *Distribution of immunophenotypic and cytogenetic subgroups of acute childhood leukemia in the NCCLS[a] and the UKCCS*

| Immunophenotype/ cytogenetic subgroup | Current study NCCLS | | | | | UKCCS[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Age | | | Gender | | Age | | | Gender | |
| | <2 | 2–5 | 6–14 | Female | Male | <2 | 2–5 | 6–14 | Female | Male |
| B-cell ALL | 15 | 86 | 40 | 69 | 72 | 109 | 579 | 263 | 417 | 534 |
| (141/951)[c] | (10.6%) | (61.0%) | (28.4%) | (48.9%) | (51.1%) | (11.5%) | (60.9%) | (27.6%) | (43.8%) | (56.2%) |
| T-cell ALL | 0 | 3 | 11 | 4 | 10 | 8 | 50 | 79 | 47 | 90 |
| (14/137) | (0.0%) | (21.4%) | (78.6%) | (28.6%) | (71.4%) | (5.8%) | (36.5%) | (57.7%) | (34.3%) | (65.7%) |
| AML | 4 | 5 | 23 | 12 | 20 | 71 | 58 | 121 | 116 | 134 |
| (32/250) | (12.5%) | (15.6%) | (71.9%) | (37.5%) | (62.5%) | (28.4%) | (23.2%) | (48.4%) | (46.4%) | (53.6%) |
| t(12;21) | 3 | 34 | 4 | 17 | 24 | 6 | 95 | 38 | 65 | 74 |
| (41/139) | (7.3%) | (82.9%) | (9.8%) | (41.5) | (58.5%) | (4.3%) | (68.3%) | (27.3%) | (46.8%) | (53.2%) |
| Hyperdiploid | 5 | 35 | 16 | 31 | 25 | 29 | 271 | 123 | 182 | 241 |
| (56/423) | (8.9%) | (62.5%) | (28.6%) | (55.4%) | (44.6%) | (6.9%) | (64.1%) | (29.1%) | (43.0%) | (57.0%) |

[a] NCCLS, Northern California Childhood Cancer Center Study; UKCCS, United Kingdom Childhood Cancer Study; ALL, acute lyphoblastic leukemia; AML, acute myeloid leukemia.

[b] UKCCS (46), B-cell ALL cases from the UKCCS ($n$ = 951) include common ALL and pro-B ALL.

[c] Numbers shown denote cases in the current study NCCLS/number of cases in the UKCCS within each row subtype. One ALL case listed in Table 1 could not be classified further; hence the total number of ALL here for the NCCLS is 155.

intensity. *GAPDH*-cycling parameters were preheated at 94°C for 2 min, then 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The reaction was repeated for 33 cycles. Using the normalized cDNA as a template, *FHIT* transcripts were amplified with previously described primers 5RT-F/3D2 (GCTCTTGTGAATAGGAAACC-sense, TCACTGGTTGAAGAATACAGG-antisense) and cycling conditions (95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 38 cycles; 33). This assay amplified a 532 bp *FHIT* transcript spanning exon 5 to exon 10. The *GAPDH* and *FHIT* PCR products were run on 3% and 2% agarose gel, respectively, and visualized by ethidium bromide staining.

Western blot analysis was performed as described previously (45); briefly, protein was extracted from leukemia cell lines and representative primary leukemia bone marrow samples using Mammalian Protein Extraction Reagent (PIERCE, Rockford, IL) with additions of 150 mM sodium chloride and Halt Protease Inhibitor Cocktail (PIERCE), and quantified with bicinchoninic acid protein assay kit (PIERCE) according to the manufacturer's instructions. One hundred $\mu$g of cell extract were electrophoretically separated on a 4–20% SDS-polyacrylamide gel with 150 V for 50 min and transferred to nitrocellulose filter under 100 V for 2 h at 4°C. The membrane was blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 for 2 h at room temperature, and the blot was then incubated overnight at 4°C with anti-FHIT rabbit antibody (Zymed Laboratories, South San Francisco, CA) diluted 1:500 in PBS containing 0.1% Tween 20 and 2.5% nonfat milk. After extensive washing with PBS containing 0.1% Tween 20, the filter was incubated for 1 h with goat antirabbit IgG horseradish peroxidase conjugate (Zymed) diluted 1:2000 and washed with PBS containing 0.1% Tween 20. The immunoreactive bands were visualized with enhanced chemiluminescence detection reagent (Amersham, Arlington Heights, IL) as described by the manufacturer. The quality of the protein was assessed by incubating the filter with antitubulin antibodies instead of anti-FHIT antibodies.

**5-aza-dC Treatment and RT-PCR.** HL-60, Blin, Reh, Molt-4, and Jurkat leukemia cell lines were maintained in culture medium with and without 0.5–1.0 $\mu$M 5-aza-dC (Sigma) for 6 days. RNA extraction and RT-PCR were performed as described above.

**FISH Detection of t(12;21) and Hyperdiploidy.** The FISH method applied in this study was designed for detecting *TEL-AML1* fusion genes derived from t(12;21) and high hyperdiploidy (>50 chromosomes, hereafter referred to as "hyperdiploid") simultaneously. Interphase FISH probes targeted to the *TEL* and *AML1* genes and the centromere of chromosome X (Vysis, Downer Grove, IL) were applied in bone marrow smears of childhood ALL patients. The *TEL* probe begins between exons 3 and 5 and extends approximately 350 kb toward the telomere of chromosome 12 and was labeled directly with SpectrumGreen. The *AML1* probe labeled directly with SpectrumOrange spans the entire gene of approximately 500 kb. The centromere probe of chromosome X was labeled with SpectrumAqua. The hybridization procedures were performed according to the manufacturer's protocols. FISH signals were viewed with a quadra-band filter (Chroma, Battleboro, VT). The t(12;21) was detected by observing the *TEL-AML1* fusion signals (yellow), whereas high hyperdiploidy was defined when the additional copies of both chromosomes 21 and X were found in the same cell, because over 90% of ALL cases that are high hyperdiploid include extra copies of both 21 and X (2). These cytogenetic characteristics were observed and confirmed by two experienced cytogeneticists.

**Statistical Analysis.** Statistical analyses were carried out using SAS analysis software. $\chi^2$ analyses were used to test for associations between *FHIT* methylation status and a variety of demographic, cytogenetic, and molecular characteristics of the patients, including age, gender, ethnicity, histological subtype of leukemia, the presence of any chromosomal translocations, t(12;21)/*TEL-AML1*, and hyperdiploidy (≥50 chromosomes in leukemic cells). Wilcoxon rank-sum test, Students *t* test, and Fisher's exact test were used to test for associations of elevated WBC counts with methylation. We also tested the association of *FHIT* methylation with a classification scheme that combined conventional and FISH cytogenetic data and incorporated immunophenotypic data to create five major subgroups of pediatric ALL, including B-cell ALL hyperdiploid (50–68 chromosomes) positive, B-cell ALL *TEL-AML1* translocation positive, B-cell ALL translocation positive & *TEL-AML1* negative, B-cell ALL hyperdiploidy & translocation negative, and T-cell ALL hyperdiploidy & translocation negative. All statistical runs were done using coded patient specimens, and all methylation analysis were carried out blind with respect to the clinical, demographic, and cytogenetic status of patients.

## RESULTS

**Study Population Demographic and Clinicopathological Characteristics.** Characteristics of the leukemia cases included in the study are shown in Table 1. To assess how well our cases represent pediatric leukemia, we compared our case series with another large and well-defined population. Table 2 shows the distribution of pediatric acute leukemias by age, gender, immunophenotype, and cytogenetic characteristics compared with the large database from the United Kingdom Childhood Cancer Study (46). The age distribution of the common B-cell leukemias (2–5 years) were very similar to the United Kingdom study as well as indicating an older age for children presenting with non-B-cell tumors. A male predominance was noted in our case series. B cell was the most common acute leukemia (71.1% United Kingdom/75.4% NCCLS), followed by AML (18.9% United Kingdom/17.1% NCCLS) and T-cell leukemia (10.2% United Kingdom/7.5% NCCLS). Among the B-cell leukemias, similar percentages of hyperdiploid tumors were found (44.5% United Kingdom/39.7% NCCLS) and t(12;21) accounted for 29.1% of B-cell leukemia in the current study compared with 14.6% in the United Kingdom study.

***FHIT* 5′ CpG Island Methylation in Cancer Cell Lines.** We analyzed 10 leukemia cell lines and found methylation of cytosine residues at CpG dinucleotides in four of them (Blin, Jurkat, Nalm, MOLT-4; Fig. 1*A*). These cell lines contained either the methylated or the unmethylated form, except the Jurkat cell line, which contained both forms (hemimethylated). No cell line that lacked both methylated and unmethylated forms was found, indicating no homozygous deletions in the locus tested. To assess the sensitivity of the MS-PCR method, DNA samples from methylated Molt-4 and unmethylated KG1A were mixed in different ratios; a single unambiguous *FHIT*-methylated band was detectable when methylated template was present at >1:32 (3%) of the total DNA but not at lower dilutions (data not shown).

**Aberrant *FHIT* Methylation in Primary Pediatric Leukemias.** We examined 190 bone marrow aspirates of primary pediatric leukemias using the MS-PCR method and found an overall *FHIT* promoter methylation frequency of 27.4% (52 of 190) and 32.1% (50 of 156) for ALL, and 6.2% (2 of 32) for AML (Table 3). MS-PCR results for representative bone marrows are shown in Fig. 1*B*. We found no methylated products among 10 peripheral blood samples from healthy individuals. The unmethylated form of *FHIT* was found in 90% of the methylated leukemia bone marrow samples, indicating some contamination with normal cells, and 100% of the unmethylated samples. No homozygous deletion in the locus tested was found. In addition to MS-PCR, which was run on all cases, we also carried out bisulfite-

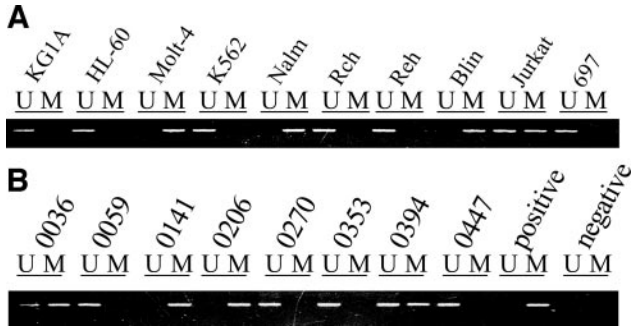


Fig. 1. Methylation analysis of fragile histidine triad (*FHIT*) promoter region by methylation-specific PCR. *A*, methylation status of 10 leukemia cell lines. Molt-4, Nalm, and Blin are methylated; Jurkat is hemimethylated; others are not methylated. *B*, *FHIT* methylation in primary bone marrow aspirates. Patients 0036, 0141, 0206, and 0394 are methylated; positive control DNA from peripheral blood of a healthy individual was treated with Sss I methylase and then bisulfite modified; negative control is a no template control. *U*, unmethylated; *M*, methylated.

Table 3 *Effects of gender, age, histologic subtype and cytogenetics on the distribution of FHIT[a] methylation in childhood leukemia*

| Patient characteristic | FHIT methylation status (%) No (unmethylated) | Yes (methylated) | *P* |
|---|---|---|---|
| Gender | | | 0.88 |
| Male | 76 (73.1) | 28 (26.9) | |
| Female | 62 (72.1) | 24 (27.9) | |
| Age (years) | | | 0.21 |
| <2 | 16 (84.2) | 3 (15.8) | |
| 2–5 | 64 (67.4) | 31 (32.6) | |
| 6–14 | 58 (76.3) | 18 (23.8) | |
| Subtype | | | 0.008 |
| ALL | 106 (67.9) | 50 (32.1) | |
| AML | 30 (93.8) | 2 (6.2) | |
| CML | 2 (100.0) | 0 (0.0) | |
| Any Translocation[b] | | | <0.0001 |
| No | 44 (58.7) | 31 (41.3) | |
| Yes | 70 (94.6) | 4 (5.4) | |
| t(12;21)[c] | | | <0.0001 |
| No | 63 (56.8) | 48 (43.2) | |
| Yes | 40 (97.6) | 1 (2.4) | |
| Hyperdiploidy[c] | | | <0.0001 |
| No | 78 (81.3) | 18 (18.8) | |
| Yes | 25 (44.6) | 31 (55.4) | |

[a] FHIT, fragile histidine trial; ALL, acute lyphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia.

[b] These analyses were limited to a total of 149 cases whose clinical cytogenetic studies were considered adequate, *i.e.* at least 20 metaphase cells were analyzed. *P* apply to comparisons using the $\chi^2$ test.

[c] These included 152 ALL cases for whom the diagnosing hospital or the University of California Berkeley performed flourescence *in situ* hybridization screening for t(12;21) and hyperdiploidy and for whom the t(12;21) and hyperdiploidy information was captured from the hospital clinical cytogenetics report. To create mutually exclusive categories, we have assumed that cases who were hyperdiploidy (≥50 chromosomes) per hospital cytogenetics are negative for t(12;21) and that T-cell ALL cases are negative for both hyperdiploidy and t(12;21) and that cases with other types of translocations are negative for both hyperdiploidy and t(12;21). These assumptions are justified by observations reported in large multicenter clinical series (1, 2).

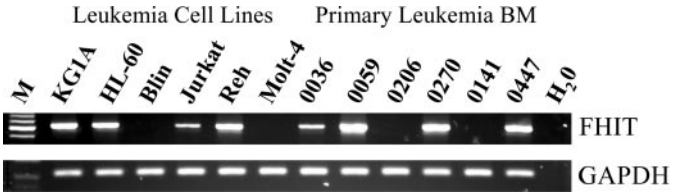


Fig. 2. Representative reverse transcription (RT)-PCR analysis. In leukemia cell lines, fragile histidine triad (*FHIT*) RT-PCR products are absent in Blin and Molt-4 and detectable in Jurkat, KG1A, HL-60, and Reh. In primary leukemia bone marrows, *FHIT* RT-PCR products are absent in patients 0206 and 0141 and detectable in 0036, 0059, 0270, and 0447. *BM*, bone marrow; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

sequencing on four randomly selected methylated (by MS-PCR) bone marrows to confirm complete C to T conversion. Each of the four methylated bone marrows showed complete conversion except for cytosines in CpG dinucleotides.

**Effect of *FHIT* Hypermethylation on Gene Expression.** By RT-PCR analysis, undetectable or very low levels of *FHIT* transcripts were found in four of six leukemia cell lines and three of six representative leukemia samples (Fig. 2; Table 4). Among the six leukemia cell lines and six primary leukemia bone marrows, we found an inverse association between *FHIT* methylation and *FHIT* transcript expression. To confirm the transcript analysis results, FHIT protein detection was performed by Western blotting. As predicted, protein product was not detectable in methylated cell lines and methylated primary leukemia bone marrows (Fig. 3; Table 4).

**Recovery of *FHIT* Transcripts after Treatment with 5-aza-dC.** Two methylated cell lines (Blin and Molt-4), two unmethylated cell lines (HL-60 and Reh), and one hemimethylated cell line (Jurkat) were treated with 0.5–1.0 $\mu$M 5-aza-dC for 6 days at which time total RNA was harvested to determine whether the locus could be reactivated with the demethylating agent. RT-PCR analysis showed that, after 5-aza-dC treatment, two methylated cell lines re-expressed mRNA of the *FHIT* gene, and hemimethylated cell line showed detectable increased mRNA production (Fig. 4).

**Cytogenetic and Patient Characteristics Correlated with *FHIT* Methylation.** As shown in Table 3, it was rare to observe *FHIT* methylation in patients who presented with tumors containing any chromosomal translocations (5.4% compared with 41.3% in patients without translocations, $P < 0.0001$). On the other hand, patients with hyperdiploid leukemia were much more likely to have *FHIT* methylation compared with other patients ($P < 0.0001$). *FHIT* methylation status was not associated with the age or gender of patients. No association was observed between *FHIT* methylation status and ethnicity (data not shown). The inclusion of immunophenotype data did not reveal any further associations within the ALL group; *FHIT* methylation was common in both CD10/CD19-positive ALL with hyperdiploidy (B cell), but it was also prevalent among the smaller

Table 4 *Relationship of FHIT[a] methylation and expression in leukemia cell lines*

| Cell lines | Classification | Methylation | Gene expression Transcripts[b] | Proteins[c] |
|---|---|---|---|---|
| Molt-4 | T-ALL | + | − | − |
| KG1A | AML | − | + | + |
| HL-60 | AML | − | + | + |
| RCH | B-ALL | − | + | + |
| Blin | B-ALL | + | − | − |
| Reh | B-ALL | − | + | + |
| Jurkat | B-ALL | +[d] | +[e] | +[e] |
| 697 | B-ALL | − | +[f] | + |
| Nalm | B-ALL | + | − | − |
| K562 | CML | − | +[g] | − |

[a] FHIT, fragile histidine triad; ALL, acute lyphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia.

[b] As detected by reverse transcription-PCR.

[c] As detected by Western blotting.

[d] Hemimethylated with both methylated and nonmethylated bands.

[e] The bands were weak.

[f] Multiple PCR bands showed products of full length and smaller molecular weight.

[g] Multiple PCR bands showed products of small molecular weight only.

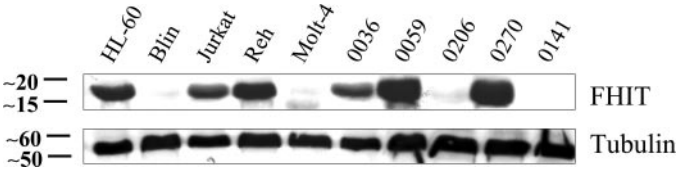


Fig. 3. Western blot analysis of fragile histidine triad (FHIT) protein expression in representative leukemia cell lines and primary bone marrow samples. After SDS-PAGE gel electrophoresis and transfer, the filter was cut between FHIT protein and tubulin protein regions based on a prestained size standard. *Upper* and *lower* portions of the filter were probed with tubulin and FHIT antibodies, respectively (in this figure, FHIT is depicted above tubulin). Tubulin probe is used to confirm equal loading of protein samples. *Left*, the size of the two marker bands for FHIT ($\sim M_r$ 17,000) and tubulin ($\sim M_r$ 55,000) are indicated.

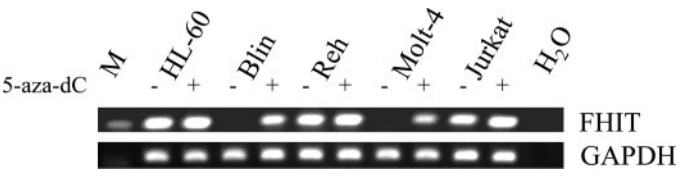


Fig. 4. Reverse transcription (RT)-PCR analysis of re-expression of the fragile histidine triad (*FHIT*) transcripts by 5-aza-dC treatment in five leukemia cell lines; (−) no treatment, (+) 5-aza-dC treated. In *farthest left lane*, M shows molecular weight markers. Methylated Blin and Molt-4 show recovery of transcription after treatment, unmethylated HL-60 and Reh show no or minimal change, and moderate increase in transcript is shown for the hemimethylated Jurkat cell line. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 5 *FHIT*[a] *methylation by immunophenotype and cytogenetic subgroups of childhood leukemia*

| FHIT methylation status | B cell ALL hyperdiploid (50–68 chr) positive | B cell ALL TEL-AML1 translocation positive | B-cell ALL translocation positive[b] & TEL-AML1 negative | B cell ALL hyperdiploid & translocation negative | T cell ALL hyperdiploid & translocation negative | Myeloid leukemia[c] | Other[d] | Total |
|---|---|---|---|---|---|---|---|---|
| Methylated | 31 (55.4%) | 1 (2.4%) | 1 (7.7%) | 11 (37.9%) | 5 (38.5%) | 2 (5.9%) | 1 | 52 |
| Unmethylated | 25 (44.6%) | 40 (97.6%) | 12 (92.3%) | 18 (62.1%) | 8 (61.5%) | 32 (94.1%) | 3 | 138 |
| Total | 56 | 41 | 13 | 29 | 13 | 34 | 4 | 190 |
| | | | | $\chi^2$: 47.7; $P < 0.0001$[e] | | | | |

[a] FHIT, fragile histidine triad; ALL, acute lyphoblastic leukemia; AML, acute myeloid leukemia.

[b] Any translocation other than TEL-AML1, includes nine recurrent [t(1;19)(q23;p13), t(1;19)(q21;p13), t(9;22)(q34;q11), ins(4;11)(q21;q13q23), t(8;14)(q24;q23)], and four nonrecurrent translocations [t(8;14)(q34;q32), t(3;9)(p10;q10), t(9;20)(q12;q11.2), t(12;?) not TEL/AML1] see Mitelman F, Johansson B, and Mertens, editors Mitelman database of chromosome aberrations in cancer (the November 2003 version of the database was accessed and searched on 02/10/04). Available from: http://cgap.nci.nih.gov/Chromosomes/Mitelman.

[c] 32 acute myeloid leukemias, 2 chronic myeloid leukemias; both methylated leukemias were AML.

[d] Other: 1 T-cell ALL with translocation, 3 unknowns due to missing cytogenetics and/or flow cytometry data, i.e. 152 total ALL are listed of the total of 156.

[e] $\chi^2$, tests the distribution of the entire table.

group of ALLs with T-cell markers (*e.g.*, CD2, CD4, CD7, and CD8; Table 5; Fig. 5). The most positive predictive variables for *FHIT* methylation appear first to be lymphocytic *versus* myeloid lineage and then the karyotypic features of pediatric ALL and specifically the absence of translocation or other detectable structural alterations.

We examined age and WBC counts at diagnosis within subgroups of pediatric leukemia. No significant differences in age at diagnosis by *FHIT* methylation status were observed. Higher median and mean WBC counts were observed among the methylated T- and B-cell ALLs that were negative for translocation and hyperdiploidy compared with unmethylated cases in this same subtype; however, the differences were not statistically significant. For example, a median value of $31.5 \times 10^6$/ml *versus* $14.2 \times 10^6$/ml WBC were observed for methylated and unmethylated cases, respectively ($P > 0.05$). Similarly, the mean WBC count was higher among methylated cases (93.9 *versus* $32.9 \times 10^6$/ml), but this difference was also only borderline significantly different ($P = 0.06$; Students *t* test). Significantly higher WBC counts at diagnosis were associated with *FHIT* methylation among hyperdiploid cases (median WBC count $11.6 \times 10^6$/ml in methylated *versus* $4.9 \times 10^6$/ml in unmethylated leukemias; $P = 0.046$). Mean WBC counts were also higher in methylated ($17.5 \times 10^6$/ml) *versus* unmethylated ($7.5 \times 10^6$/ml) hyperdiploid cases ($P = 0.03$; Students *t* test). It is important to note that most hyperdiploid patients do not have elevated WBC counts at diagnosis. In our study, nine patients presented with $\geq 20 \times 10^6$ leukocytes/ml, and importantly, eight of these were *FHIT* methylation positive ($P = 0.03$; Fisher's exact test). Only 4 of 56 hyperdiploid cases presented with $>40 \times 10^6$ WBCs/ml, and all of these were found to be *FHIT* methylation positive. We also had access to data on *p15INK4b* methylation in this series; 44 of 190 (23.2%) cases were p15 methylation positive, but no association was found between *p15INK4b* and *FHIT* methylation status (data not shown).

## DISCUSSION

The current study emphasizes the role of aberrant methylation in the inhibition of *FHIT* gene expression in leukemia. We compared *FHIT* methylation and loss of *FHIT* transcripts by RT-PCR assay and found a strong concordance between methylation and lack of expression among all leukemia cell lines. Results of Western blot analyses showed a similar match between *FHIT* methylation and FHIT expression, except for cell line K562, which was not methylated but also did not express FHIT protein. We looked at the mRNA patterns of K562 and found only aberrant transcripts, indicating deletion or rearrangements may be the mechanism for *FHIT* inactivation in this cell line. Additional evidence that supports the importance of *FHIT* methylation in transcriptional silencing is the reexpression of the *FHIT* gene after treatment with 5-aza-dC, a known demethylating agent. As

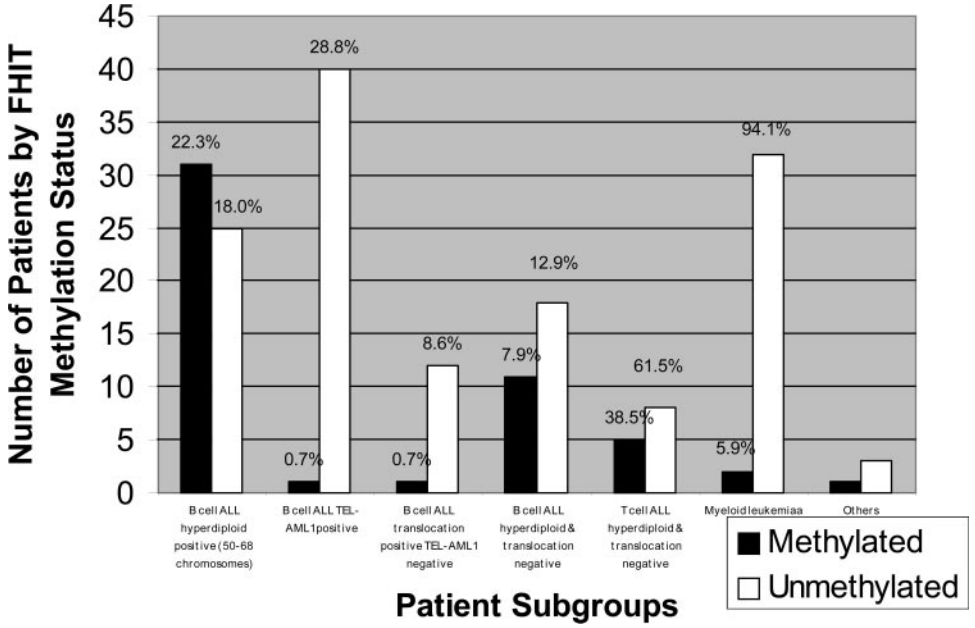


Fig. 5. Fragile histidine triad (*FHIT*) methylation in immunophenotype and cytogenetic subgroups of childhood leukemia. Histogram originate depicts the numbers of primary leukemia cases that demonstrated *FHIT* methylation (*black*) or unmethylated CpG regions (*white*) as described in the "Materials and Methods." Subgroups of cases were created according to conventional cytogenetic and fluorescence *in situ* hybridization (FISH) analyses and immunophenotype (cluster of differentiation markers). The percentages indicated above histograms apply to the percent of all B-cell acute lymphoblastic leukemia (ALL), all T-cell ALL, and all myeloid leukemias.

predicted, these findings are in agreement with the results found in esophageal squamous cell carcinoma and lung and breast cancers (33, 34). Taken together, our results demonstrate that promoter aberrant methylation of *FHIT* is an important mechanism for inactivation of this tumor suppressor gene in hematological malignancies.

The most dramatic findings of this study are the common occurrence of *FHIT* methylation among specific subtypes of primary pediatric ALL and the uncommon occurrence of *FHIT* methylation in myeloid leukemias. Methylation of *FHIT* occurred in 27.4% of our pediatric leukemia patients but was most common among the hyperdiploid ALL subgroup (55.4% methylated). In marked contrast, only 2.4% of t(12;21)/*TEL-AML*-positive cases were methylation positive, and interestingly the B-cell ALLs with translocations other than t(12;21)/*TEL-AML* also showed a low methylation rate. Because our case series was extensively characterized, we were able to combine immunophenotypic data with the conventional and molecular cytogenetic data for each patient. Considering all available patient characteristics, we found that irrespective of immunophenotype, those ALL cases that did not have detectable structural chromosomal alterations were most likely to demonstrate *FHIT* methylation, and the lowest rates were observed in ALL with translocations or other structural chromosomal changes. Within the hyperdiploid cases, the data also indicated a trend toward higher WBC counts with *FHIT* methylation. In contrast, only 5.9% of pediatric AML demonstrated *FHIT* methylation.

The marked association of *FHIT* methylation with hyperdiploid ALL suggests an etiological role of *FHIT* in this type of leukemia. A unique mechanism involving a single aberrant mitotic division that leads to a highly nonrandom aneuploidy has been proposed in the evolution of the high hyperdiploid subtype of ALL (47). It is unknown whether this mitotic event precedes or follows the methylation of *FHIT* in hyperdiploid ALL. Hyperdiploidy is associated with a favorable survival outcome among ALL patients (48), and possibly relevant to this is the observation that a large proportion of hyperdiploid leukemic blasts display a high rate of spontaneous apoptosis (49). Interestingly, *FHIT* has been implicated in apoptosis (32, 50) and in the metabolism of $Ap_3A$ (51). IFNs and other cytokines effectively induce apoptosis in hyperdiploid and other leukemias (29) and have been shown to also induce $Ap_3A$ levels (27, 28, 52). In this regard, our finding of higher WBC counts at diagnosis among *FHIT*-methylated cases may be indicative of leukemic cells that are defective in some *FHIT*-related apoptotic mechanism or pathway.

A similarly high rate of *FHIT* methylation (*i.e.,* 37.9%) was also observed among ALL cases that exhibited the normal complement of chromosomes (*i.e.,* 46 XX, 46 XY). Although conventional cytogenetic analyses may fail to detect hyperdiploid leukemic cells in bone marrow aspirates, we also screened these "normal" cases with a sensitive and specific FISH assay. This makes it unlikely that we have misclassified this latter subgroup. On the basis of these observations, we interpret our data to indicate that the strongest association of *FHIT* methylation is with pediatric ALL of either T- or B-cell origins that do not contain structural chromosomal alterations and instead display normal or hyperdiploid karyotypes.

Because so few AML cases displayed *FHIT* methylation, our data suggest that transcriptional silencing of *FHIT* by an epigenetic mechanism may be relatively uncommon in pediatric AML. This conclusion must be considered tentative because we did not assess protein expression; however, previous studies indicate a close correlation of *FHIT* methylation with loss of *FHIT* gene expression (34). In our study, gene expression tracked exquisitely with methylation in leukemia primary samples and cell lines (Figs. 2–4). If *FHIT* is not down-regulated in pediatric AML, this would contrast with previous results in adult AML that show frequent loss of *FHIT* expression. Even in adult myeloid leukemia, it is interesting that previous studies suggest that structural chromosomal alterations may be inversely correlated with *FHIT* abnormalities, as we found in our series. For example, in previous studies of Philadelphia chromosome (Ph)-positive chronic myelogenous leukemia, intact (*i.e.,* normal) *FHIT* transcripts were observed in all cases (14). Fewer than 4% of Ph-positive chronic myeloid leukemias showed low levels of FHIT protein by Western blot analysis (18).

Decreased or absent *FHIT* expression could arise through either epigenetic or genetic mechanisms affecting the *FHIT* locus. In one study, about half of B-cell and all T-cell ALLs examined demonstrated reduced FHIT protein expression (16). Loss of FHIT was also very common in AML (17, 21). These previous studies did not specifically examine pediatric leukemia as we have in our series; to our knowledge, ours is the first study of *FHIT* methylation in pediatric leukemia. In a recent study comparing pediatric and adult ALL, the prevalence of methylation of a panel of gene loci (*ER*, *MDRT*, *p15*, *C-ABL*, *CD10*, *p16*, *p73*) was found to be very similar in 16 pediatric and 61 adult ALL (11). Although not a focus of the current study, we found 23% of pediatric ALL to demonstrate *p15INK4b* methylation, which is very similar to the previous report in childhood ALL (*i.e.,* 25% *p15INK4b* methylation positive; Ref. 11). We found no correlation of *FHIT* with *p15INK4b* methylation in our study, and hence *FHIT* probably represents a class of methylation targets distinct from those examined previously. Additional studies of *FHIT* methylation and expression in adult ALL would be useful.

Interestingly, human tumor suppressor gene *FHIT* is located on the short arm of chromosome 3p, a region that harbors many other potential tumor suppressor genes (53). One of most important discoveries involving putative tumor suppressors in this region is the importance of tumor-acquired promoter hypermethylation as an epigenetic mechanism for inactivating the expression of these genes. *DUTT1* (*ROBO1*) at 3p12, *RASSF1A* at 3p21.3, *BLU* at 3p21.3, *SEMA3B* at 3p21.3, *HYAL1* at 3p21.3, *CACNA2D2* at 3p21.3, *RARβ2* at 3p24, and *VHL* at 3p25.3 have been found to undergo hypermethylation and are associated with absent or reduced expression in various human malignancies (34, 54–58). Three of those genes, *RASSF1A*, *RARβ2*, and *FHIT*, have been shown to be involved in human leukemia (55, 59). Therefore, methylation of genes residing in the 3p region should be further examined in childhood leukemia. It will be of interest to see whether a concordant pattern of methylation in the 3p region is associated with the specific cytogenetic subtypes of ALL.

DNA hypermethylation, affecting the *p15INK4b*, *p16INK4a*, *RB*, *p73*, *NNAT*, and calcitonin loci in childhood leukemia have been reported (3–5, 60). Links between aberrant methylation and clinicopathological features were observed, such as reduced survival and higher relapse rate (4, 60, 61). Within ALL high peripheral WBC count at diagnosis is associated with a relatively worse survival outcome. *FHIT* methylation should be investigated further as a prognostic marker because we found that *FHIT* methylation was more common among the hyperdiploid cases that had the highest WBC counts at diagnosis. Moreover, the mechanisms leading to aberrant methylation in leukemia are only poorly understood and deserve much more intensive investigation. It has been proposed that in some cytogenetic subtypes of leukemia, aberrant CpG methylation is induced by abnormal chromosomal fusion proteins, which recruit DNA methyltransferases to target specific promoters (59). Because we found *FHIT* methylation to be inversely associated with chromosomal translocations, it is unlikely that this mechanism is responsible for targeting aberrant methylation within the *FHIT* locus. Additional studies are also necessary to determine whether *FHIT* methylation could be a useful marker of etiologically important subgroups of pediatric leukemias. Comparing environmental exposures of leukemia cases or their parents according to the *FHIT* methylation status of the child's diagnostic bone

marrow may be one way to gain insights into epigenetic mechanisms operating in some subtypes of pediatric leukemia.

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