FHIT promoter DNA methylation and expression analysis in childhood acute lymphoblastic leukemia

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Abstract. Fragile histidine triad (FHIT) is a tumor suppressor gene, which is involved in several malignancies. Epigenetic alterations in *FHIT* have been hypothesized to contribute to tumorigenesis. The present study aimed to examine DNA promoter methylation and gene expression levels of *FHIT* in childhood acute lymphoblastic leukemia (ALL), in a sample of Iranian patients. The promoter methylation status of *FHIT* was analyzed in 100 patients diagnosed with ALL and 120 healthy control patients. mRNA expression levels were assessed in 30 new cases of ALL compared with 32 healthy controls. Hypermethylation of the *FHIT* promoter was significantly more frequent in patients with ALL than in healthy controls (OR=3.83, 95% CI=1.51-9.75, P=0.007). Furthermore, *FHIT* mRNA expression levels were significantly reduced in childhood ALL patients compared with healthy controls (P=0.032). The results of the present study revealed that dysregulation of the *FHIT* gene may contribute to the pathogenesis of childhood ALL. Future studies investigating a larger sample population with greater ethnic diversity would be beneficial, to confirm the results from the present study.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer (1). In all cases of childhood leukemia, ~80% are ALL, which represents ~25% of cancers in children diagnosed <15 years of age in the United States in 2014 (2). Although the etiology of ALL is largely unknown, it has been proposed that multiple gene alterations, including inactivation of tumor suppressor genes, activation of proto-oncogenes and chromosomal rearrangements, in addition to gene-environmental interplay, are involved in disease development (1,3,4). Chromosomal abnormalities, including homozygous deletions and loss of heterozygosity, are among the most common characteristics of human tumors. The fragile histidine triad (*FHIT*) gene is located on the short arm of human chromosome-3 (3p14.2) which is a major site of chromosomal rearrangement (5).

FHIT protein has previously been proposed to act as a tumor suppressor (6-8). However, the exact mechanisms by which FHIT mediates its suppressive functions are not well understood. Several researchers have indicated that restoration of *FHIT* expression suppresses tumorigenicity, and transfection of *FHIT* in FHIT-deficient human cancer cells appears to induce apoptosis and inhibit cell growth (8-11). DNA hypermethylation may occur in genes involved in the cell cycle, DNA damage repair and signaling pathways. DNA methylation is a well-studied epigenetic modification, which significantly contributes to leukemia development (12). Several studies have demonstrated an association between *FHIT* hypermethylation and an increased risk of non-small-cell lung carcinoma (NSCLC) (13), breast cancer (14,15), cervical cancer (16), hepatocellular carcinoma (17) and thyroid carcinoma (18). Furthermore, downregulation of *FHIT* expression has been observed in acute lymphoblastic leukemia (ALL) (19), gastric cancer (20), colon adenocarcinoma (21), nasopharyngeal carcinoma (22) and colorectal cancer (23).

It has been proposed that the loss of normal FHIT function may be involved in the pathogenesis of several human leukemias, and the aberrant expression of *FHIT* is specific and frequent in leukemia samples (24,25). The frequent loss of *FHIT* expression in ALL suggests that inactivating alterations at the *FHIT* locus may contribute to the development of leukemias (26). The present study aimed to assess promoter methylation status and expression levels of the *FHIT* gene in childhood ALL.

Materials and methods

Patients. A case-control study was conducted, in which 100 children diagnosed with ALL (54 male, 46 female; age, 5.5±3.6 years) and 120 age and sex matched healthy controls (58 male, 62 female; age, 5.6±2.9 years) in Zahedan, Iran were

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Table I. Primer sequences for MS-PCR and RT-qPCR.

Gene	Primers (5'-3')	Amplicon size (bp)
Nested	F: TTGATGGATTAAGTTAGGGATTGTAA R: CCCCTACCTTCCAAAATATTAACA	623
FHIT M	F: TTTTCGTTTTTGTTTTTAGATAAGC R: AAAAATATACCCACTAAATAACCGC	157
FHIT U	F: TGGTTTTTGTTTTTGTTTTTAGATAAGT R: AAAATATACCCACTAAATAACCACC	159
FHIT RT-qPCR	F: ACCTGCGTCCTGATGAAGTG R: CGTGAACGTGCTTCACAGTC	144
GAPDH RT-qPCR	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	226

MS-PCR, methylation specific polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; *FHIT*, fragile histidine triad; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

enrolled. All subjects were selected from the Ali-Ebne-Abitaleb Hospital, Zahedan University of Medical Sciences (Zahedan, Iran) between February 2013 and May 2014. Details of the study design and enrolment process have been previously described (27-29). Analysis of DNA methylation in the *FHIT* promoter was performed in 100 ALL cases and 120 healthy age and sex matched cases. Analysis of *FHIT* mRNA expression levels was conducted in 30 new cases of childhood ALL (20 males and 10 females; mean age, 5.5±3.4 years; age range, 1-15 years) and 32 healthy age and sex matched children (15 males and 17 females; mean age, 5.0±4.1 years; age range, 1-15 years). The subjects were selected from the Ali-Ebne-Abitaleb Hospital, Zahedan University of Medical Sciences (Zahedan, Iran) between February 2013 and May 2014. The local ethics committee of Zahedan University of Medical Sciences (Zahedan, Iran) approved the studies and written informed consent was obtained from parents of cases and controls.

Promoter methylation. A methylation specific PCR (MSP) technique was used for determination of promoter DNA methylation status. Whole blood samples (2 ml) were collected in EDTA-containing tubes, and genomic DNA was extracted using the salting out method as described previously (30). DNA was modified by sodium bisulfite using the CPGenome™ Direct Prep Bisulfite Modification kit (Merck KGaA, Darmstadt, Germany). An MSP was established for the evaluation of *FHIT* promoter methylation. The primer sequences are listed in Table I. To each 0.2 ml PCR reaction tube, 1 μl bisulfite-converted DNA, 1 μl of forward and reverse primer (10 μM) and 17 μl dH2O were added with HotStart PCR PreMix (AccuPower, Bionner Corp, Daejean, South Korea). The PCR conditions were set as follows: 95˚C for 5 min, 30 cycles of 95˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 30 sec, with a final extension step of 72˚C for 5 min. The PCR product (623 bp) was used as a template for MSP. MSP was performed using two pairs of primers; one pair specific for methylated and the other for unmethylated template (Table I). In each 0.2 ml PCR reaction tube, 0.5 μl nested PCR product, 1 μl of each primer (10 μM) and 10 μl 2X Prime Taq Premix (GeNet Bio, Daejean, South Korea) and 7 μl dH2O were added. MSP conditions included an initial denaturation step at 95˚C for 5 min, followed by 30 cycles of 30 sec at 95˚C, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec, with a final extension step at 72˚C for 5 min. The methylated and unmethylated PCR products were 157 and 159 bp, respectively (Fig. 1).

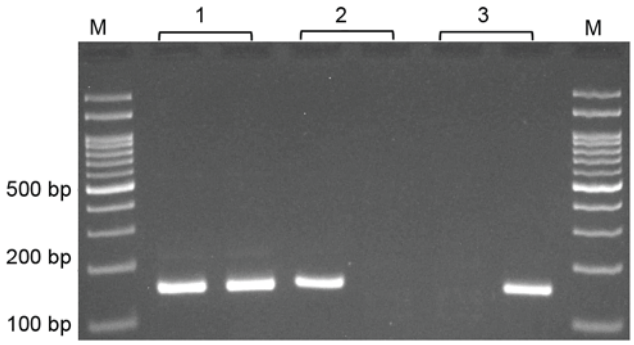

Figure 1. Electrophoresis pattern of promoter methylation of *FHIT* using methylation specific polymerase chain reaction. Lane 1, MU; lane 2, MM; lane 3, UU. M, DNA marker; FHIT, fragile histidine triad; MU, methylated unmethylated; MM, methylated methylated; UU, unmethylated unmethylated.

Analysis of FHIT gene expression by RT-qPCR in patients with ALL and healthy controls. Total mRNA extraction from fresh whole blood was conducted using the commercially available kit, GeneJET RNA Purification kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA synthesis was performed using the AccuPower CycleScript RT PreMix kit (Bioneer Corp., Daejeon, Korea) in a final volume of 20 μl according to the manufacturer's protocol. The mRNA expression levels of *FHIT* and an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were detected by qPCR using SYBR Green 2X RealQ Plus master mix (Amplicon; Bio, Korea; www.amplicon.com) on the ABI quantitative Real-Time PCR

Table II. Promoter DNA methylation of the *FHIT* gene in patients with ALL and controls.

Methylation status	Cases, n (%)	Controls, n (%)	OR (95% CI)	P-value
Absent	12 (12.0)	30 (25.0)	1.00	
Partial	65 (65.0)	75 (62.5)	2.17 (1.03-4.58)	0.050[a]
Present	23 (23.0)	15 (12.5)	3.83 (1.51-9.75)	0.007[a]
Partial + present	88 (88.0)	90 (75.0)	2.44 (1.18-5.08	0.016[a]

[a]P<0.05. OR, odds ratio; CI, confidence interval.

system (Applied Biosystems, Thermo Fisher Scientific, Inc.). All primer sequences are presented in Table I. To each 0.2 ml PCR reaction tube, 1 μl of cDNA, 1 μl of forward and reverse primers, 10 μl 2X RealQ Plus Master Mix Green High Rox (Amplicon Bio, Korea) and 7 μl dH_2O was added. The PCR conditions were set as follows: 95˚C for 6 min, 40 cycles of 95˚C for 40 sec, 63˚C for 40 sec, and 72˚C for 35 sec, with a final extension step of 72˚C for 10 min. Results are presented as gene expression fold change of ALL samples compared with controls, according to the $2^{-\Delta\Delta Cq}$ method (31).

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Data were analyzed using an independent sample t-test for continuous data and Fisher's exact test or χ^2 test for categorical data. The odds ratio (OR) and 95% confidence intervals (CI) were calculated from logistic regression analysis to identify the associations between methylation status and ALL. P<0.05 was considered to indicate a statistically significant difference.

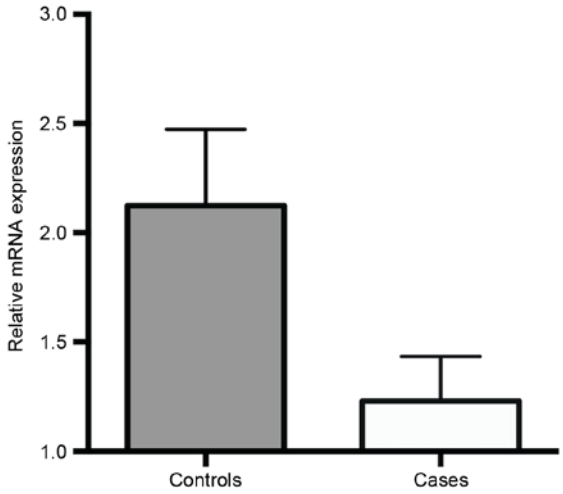

Figure 2. Relative mRNA expression of *FHIT* in patients with childhood ALL and controls. mRNA expression levels of *FHIT* were significantly downregulated in ALL (P=0.032). Statistical analysis was performed using an unpaired t-test with Welch's correction. FHIT, fragile histidine triad; ALL, acute lymphoblastic leukeamia.

Results

The case-control study was conducted on 100 childhood ALL cases (54 male, 46 female; age, 5.5±3.6 years) and 120 healthy children (58 male, 62 female; age, 5.6±2.9 years). No significant differences were observed between groups regarding sex and age (P=0.419 and 0.761, respectively). Table II presents the promoter DNA methylation status of the *FHIT* gene in patients with ALL and controls. The frequency distribution of non-methylation, partial methylation and hypermethylation in cases and controls were 12.0, 65.0, and 23.0%, and 25.0, 62.5, and 12.5%, respectively. Hypermethylation of the *FHIT* gene was significantly more frequent in children with ALL than in healthy controls (OR=3.83, 95% CI=1.51-9.75, P=0.007). *FHIT* mRNA expression levels in ALL cases and controls are presented in Fig. 2. These results revealed that the gene expression of *FHIT* was significantly downregulated in ALL cases compared with that in healthy children (1.231±0.204 vs. 2.124±0.349; P=0.032).

Discussion

FHIT is a candidate tumor suppressor gene in multiple types of cancer, although the mechanism of tumor suppression by *FHIT* is not fully understood. Several investigations have revealed that FHIT overexpression leads to the activation of the two main apoptotic pathways, the extrinsic caspase-8 pathway and the intrinsic mitochondrial pathway (32-34). The inactivation of programmed cell death may lead to tumorigenesis (35).

In the present study, hypermethylation of the *FHIT* gene was demonstrated to be significantly more frequent in children with ALL than in healthy controls. Furthermore, the gene expression of *FHIT* was significantly lower in patients with childhood ALL compared with healthy controls. The results of the present study indicated that epigenetic modifications and altered gene expression of *FHIT* in childhood ALL, may contribute to the development of this disease.

In agreement with results of the present study, Malak *et al* (19) demonstrated that the expression of *FHIT* was significantly lower in childhood ALL compared with healthy children. In addition, Chen *et al* (36) reported that mRNA expression of *FHIT* was significantly lower and methylation frequency of *FHIT* was significantly higher in ALL samples compared with controls.

Previous studies have provided evidence that DNA methylation is the most commonly detected alteration in adult ALL (37-40). Genome-wide DNA methylation profiles are commonly altered in pediatric ALL (41). It has been demonstrated that hypermethylation of multiple genes may be involved in the relapse of childhood ALL (42). Suppression of FHIT may be involved in the development of rearranged acute lymphoblastic leukemia, an aggressive type of ALL (43).

A meta-analysis performed by Wu *et al* (13) indicated that *FHIT* hypermethylation, which induces the inactivation of the *FHIT* gene, is associated with an increased risk and adverse clinical outcome of NSCLC. It has been demonstrated that *FHIT* promoter hypermethylation is associated with the development of breast cancer and certain poor prognostic features of the disease (14). FHIT may be a potential drug target for the development of demethylation treatment for patients with breast cancer (44). Furthermore, hypermethylation of the *FHIT* promoter was previously demonstrated to be involved in the development and transformation of fetal mesenchymal stem cells into F6 tumor cells (45). In conclusion, the results of the present study suggested that hypermethlation and mRNA downregulation of *FHIT* gene may be involved in the development of pediatric ALL. Further studies are required to reveal the exact role of FHIT on ALL risk.

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