ORIGINAL ARTICLE

Study of Fragile Histidine Triad (*FHIT*) gene Promoter DNA Methylation in Acute Lymphoblastic Leukemia (ALL) patients

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ABSTRACT

Background: The fragile histidine triad (*FHIT*) gene has earlier been projected as a tumor suppressor in many disease mechanisms including acute lymphoblastic leukemia (ALL). DNA methylation in the promoter region of *FHIT* gene has been linked with gene silencing, and hence its inactivation is responsible in the pathogenesis of many kinds of human malignancies. The aim our study was to evaluate the frequency of *FHIT* hypermethylation in a cohort of ALL cases from Kashmir region (North India).
Methods: *FHIT* gene hypermethylation was investigated in a cohort of 66 confirmed ALL patients at diagnosis by methylation specific PCR (MS-PCR).
Results: Our results showed *FHIT* promoter methylation was significantly higher in ALL patients with a frequency of 80% (53 of 66). *FHIT* gene promoter methylation was seen higher in males 84% as compared to females75%. Older age group (≥18years) patients showed higher 87% *FHIT* gene hypermethylation as compared to 78% in lower age group <25 years. Around 94% of subjects harboring hypermethylated FHIT gene were having higher WBC count as compared to cases with lower WBC count ($p>0.05$).
Conclusion: We conclude that *FHIT* gene hypermethylation is highly implicated in ALL cases and seems as an important event in the ALL pathogenesis.
Keywords: *FHIT* gene, Promoter Methylation, Methylation Specific PCR, Acute Lymphoblastic Leukemia
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INTRODUCTION

ALL is a heterogeneous leukemia mostly based on genetic alterations of lymphoid progenitor cells in the bone marrow, blood and extramedullary sites. ALL is the most frequent type of childhood cancer that accounts for ~ 25% of all hematological malignancies diagnosed in children till 14 years of age[1]. In our region, Kashmir (North India), ALL cites as the 5th among commonly occurring cancers and has a frequency of around 9.9% with most common cases in ALL[2]. Among ALL cases, B-ALL, comprises approximately 80-85% as compared to rest of T-ALL cases[3]. Although, a number of available reports from different researchers have found different markers to classify ALL but results are diverse rather than perfect. Like other tumors, ALL is the outcome from the amassing of genomic abnormalities that affect normal control of cellular growth[4]. Key pathway of leukomogenesis in ALL is inactivation of tumor suppressor genes and among them, FHIT gene located on the short arm of human chromosome-3 (3p14.2) is the vital genomic derangement that has earlier been projected as a tumor suppressor in number of disease mechanisms including ALL -7. Any change in the functional sites of this gene could lead to its

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dysregulation among various such processes DNA methylation at CpG sites in the promoter region of *FHIT* has been associated with gene silencing, and hence its inactivation that cause subsequent disease progression wherever fhit protein plays it role. Process of hypermethylation in the promoter region of FHIT gene is a frequent process and has vital role in the pathogenesis of many kinds of human malignancies -10 .

DNA methylation is a well-studied epigenetic modification, which contributes to leukemia development. It has been verified that there is an important involvement between *FHIT* hypermethylation and an enlarged risk of different malignancies including non-small-cell lung carcinoma[12], breast cancer 4, cervical cancer 5, hepatocellular carcinoma[16] and thyroid carcinoma[17]. Considering the above evidence, we hypothesize that that the loss of normal *FHIT* role may also get dysregulated in ALL patients subsequent to *FHIT* gene hypermethylation.

Since a significant frequency of ALL exist in our population with no prominent study of *FHIT* gene across the subcontinent, the current study was under taken to investigate promoter methylation status of the *FHIT* gene in both childhood and adult ALL cases.

MATERIALS AND METHODS

Study subjects

The study was conducted in the Department of Advanced Centre for Human Genetics, SKIMS where a total of 66 confirmed, previously untreated ALL patients attending Department of Hematology and Medical Oncology, Sher-I- Kashmir Institute of Medical Sciences (SKIMS), J&K, India were included in this study. The patients diagnosed with ALL were included with their written consent. The peripheral blood samples taken were already reviewed by specialized Hematologist to confirm ALL disease and ensure standardization of classification criteria. Approximately 2-3 ml of the blood was collected in EDTA containing vials (200µl of 0.5M, pH=8.0) depending upon the stage and type of the disease from patients and from gender and age matched healthy controls that had no hematological/and or any other malignancy. The samples were stored until further processing for DNA extraction and aliquot of same samples were properly stored and preserved for protein expression at -80°C. Hemetologically confirmed ALL samples were used for hypermethylation analysis of *FHIT* gene.

The institutional ethics committee of SKIMS permitted the study protocol and written informed consent was sought from the ALL cases (Parental consent obtained in case of minor) as well as controls. The medical history of ALL patients (radiological and hematology/bone marrow reports) was recorded from personal interviews. Those patients who received previous cytotoxic chemotherapy were excluded from the analysis. Clinical information including gender, age, type of leukaemia and stage, were obtained from the review of patients/medical records.

DNA extraction

All the patient and control samples were subjected to DNA extraction by using phenol/chloroform method and DNA extraction kit (Qaigen). The purity and concentration of DNA was estimated at the absorbance of 260 and 280 nm and checked on 1% agarose gel

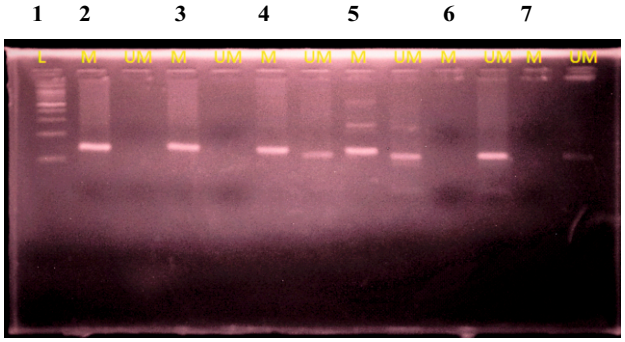
Methylation-Specific Polymerase Chain Reaction (MS-PCR) by Bisulphite DNA Modification method

Approximately 1.5-2µg of extracted DNA was treated with bisulfite modification (EZ DNA Methylation Kit,Zymo Research Corporation, USA) to convert unmethylated cytosine to uracil, while as methylated DNA remained unaffected.

MS-PCR method was performed to analyze the *FHIT* gene promoter DNA methylation status using two pairs of primers one specific for methylated and the other one for unmethylated DNA template discriminating between the two. In two tube reaction, 0.2 ml PCR reaction tubes, 1-2 µl bisulfate converted DNA,10x reaction buffer 2.5 µl, MgCl2 2.0 µl (25 mM), 0.5 µl of each primer (10 µM), 0.5 µl Taq Polymerase (Genei), dNTPs's 0.5 µl (10 µM), DMSO 1.0 µl and 15.7 µl dH2O were added. The description of the protocol methods is given in Table 1.

MS-PCR conditions included an initial denaturation step at 95˚C for 7 minutes, followed by 35 cycles of 35seconds at 95˚C, annealing at 55˚C for 35 seconds, and

extension at 72˚C for 30 seconds, with a final extension step at 72˚C for 7 minutes. The final PCR products were subjected to electrophoresis on a 3% agarose gel that was stained with ethidium bromide. The PCR products produced were 138bp and 130bp for methylated and unmethylated DNA respectively. The PCR gel bands were identified by using gel documentation system, Flourchem HD2 (Cell Bioscience, USA). The representative figure is given in Figure 1



MM, Methylated, MU: Partially methylated, UU: Unmethylated

Fig. 1:Electrophoresis pattern of the gel picture for promoter hyper methylation of *FHIT* gene

Lane 1, 100 bp DNA Marker, lane 2, MM; lane 3, MM. lane 4,MU; lane 5, MU: lane 6, UU, lane 7: UU

Statistical analysis

Statistical analysis was performed by using IBM Statistics SPSS software (Version-23).The ALL cases comparison was done using the chi square test for categorical variables, like gender and age, of the demographic variables. Statistical analysis was performed by using IBM Statistics SPSS software (Version-23). Statistical significance was set at the level of $p< 0.05$.

RESULT

This study included 66 cases of ALL patients which were analyzed for FHIT promoter hyper-methylation. Gender wise, males show maximum representation (58%) while on the basis of dwelling higher number of cases was from rural areas (83%). As a common presentation of ALL, majority of the cases showed substantially higher WBC count (74%). The patients comprised of 38 males (58%) as compared to 28 females (42%). The ratio of male: female observed was 1:0.74. More cases were seen in age group <18 years (n=61; 92.4%) than in ≥18 years (n=5; 7.6%). Based on immunophenotypes observation, there were 94% (n=62) Precursor B-cell ALL cases, 3% (n=02) T-cell ALL and 03% (n=02) Mixed phenotype ALL. More cases were found in B –cell ALL (94%) than other types of ALL (Table 1). Among 66 ALL samples, fusion transcripts aggregated to 19.6% (13/66). ETV6-RUNX1 fusion transcripts were detected in 9% (6/66), 4/66 (6%)

Table 1: Primer sequences for MS-PCR

GENE	PRIMER SEQUNCE	Annealing Temperature	APLICON SIZE (bp)
***FHIT* M F**	TTTTCGTTTTTGTTTTTAGATAAGC	55^0C	138
***FHIT* MR**	AAAAATATACCCACTAAATAACCGC		
***FHIT* UMF**	TGGTTTTTGTTTTTGTTTTTAGATAAGT	55^0C	130
***FHIT* UMR**	AAAATATACCCACTAAATAACCACC		

Table 2: Clinico-pathological and other characteristic of acute lymphoblastic leukemia Patients.

Characteristics	Sub groups	ALL Cases, n= 66 (%)
Gender	Male Female	38(58 %) 28 (42 %)
Age	<18 years >=18 years	61(92.4 %) 5 (7.6 %)
WBC counts (10^3/µl)	<10x1000 >=10x1000	48 (73 %) 18(27 %)
Immunophenotype	T-ALL precursor-B ALL Mixed phenotype	02 (3 %) 62 (94%) 02 (3 %)
Platelet count at base line — cells/mm^3	>=150x1000 <150x1000	15(23 %) 51(77 %)
Dwelling	Urban Rural	11(17 %) 55(83 %)

Table 3: *FHIT* promoter Methylation status with respect to different parameters of ALL patients

Parameters	Total patients n=66 (%)	*FHIT* Promoter Methylation		OR	9%(CI)	p value 0.0005
		Methylated n=53(80 %)	Unmethylated n=13(20 %)			
Gender Male Female	 38(58 %) 28 (42 %)	 32(84%) 21(75 %)	 6(16%) 7(25 %)	 Ref 0.56	 0.1658-1.9081	 0.53
Age <18 years >=18 years	 61(92.3 %) 5 (7.6 %)	 50(92.4%) 03(0.56%)	 11(22 %) 2(13 %)	 Ref 0.33	 0.49-2.21	 0.2
WBC counts (103/µl) <10 x1000 >=10x1000	 48 (73 %) 18(27 %)	 36(75%) 17(94 %)	 12(25%) 1(6 %)	 Ref 5.6	 0.68-47.21	 0.09
Immunophenotype precursor-B ALL T-ALL Mixed phenotype	 62 (94%) 02 (3 %) 02 (3 %)	 49(79%) 2(100%) 2(100%)	 13(21%) 0 0			 1.0 1.0
Platelet count at base line — cells/mm^3 >=150x1000 <150x1000	 15(23 %) 51(77 %)	 10(66 %) 43(84 %)	 05(34 %) 08(16 %)	 Ref 2.6	 0.72-9.98	 0.15
Dwelling Urban Rural	 11(17 %) 55(83 %)	 8(73 %) 45(82 %)	 3(23 %) 10(18%)	 Ref 1.68	 0.37-7.51	 0.67
Fusion Transcript Positive Negative	 53(80 %) 13(20 %)	 42(79%) 11(85 %)	 11(21%) 02(15 %)	 Ref 0.69	 0.13-3.60	 1.0

showed BCR-ABL, 2/66 (3%) had E2A-PBX while 1(1.5%) had MLL-AF4 fusion transcript respectively. Majority of the fusion transcripts were found in B-cell ALL 12(92%). The clinico-pathological characteristics of the patients are given in Table2.

Our results show that *FHIT* promoter methylation was significantly higher in ALL subjects with a frequency of 80% (53) in ALL patients as against 20.0% (13) with normal methylation pattern in their promoter region (p=0.0005).

On stratification of subjects on the basis of hypermethylation status in combination with various clinico-pathological factors, we observed higher FHIT hypermethylation in cases with precursor B-cell ALL49 (79%) as compared to 13(21%) unmethylated FHIT gene sequence. In case of T-cell ALL and mixed phenotype ALL, hypermethylation was observed in only 02 cases for each group respectively. There were 13 (20%) of subjects with positive fusion transcripts as compared to 53(80%) cases with negative fusion transcripts. In 13(20%) of the subjects with positive fusion transcripts, 11(85 %) cases showed hyper-methylation as compared to 02(15 %) unmethylated cases. However in case of negative fusion transcripts, 42(79%) showed hypermethylation and 11(21%) cases were unmethylated (p>0.05).

FHIT gene promoter methylation was seen higher in males 32(84%) as compared to females 21 (75%). Older age group (≥25years) patients showed higher 13(87%) promoter methylation of *FHIT* gene as compared to 40(78%) in lower age group (<25 years) as shown in Table 2.The patients with lower platelet count <150 x 109 showed 43(84%) hypermethylation and 05(34%) were unmethylated while as in patients with higher platelet count ≥150, 10(66%) showed methylated cases and 05(34%) unmethylated. Higher 17(94%) number of subjects harboring hypermethylated *FHIT* gene were having higher WBC count as compared to those with lower WBC count 36(75%) but these differences were

not statistically significant (p>0.05) as shown in Table 2.

On stratifying subjects on the basis of methylation status in combination with various demographic and clinical-pathological factors, we could not find any significant correlation with any of the studied parameters like gender, age, place of residence, WBC count, platelet count, immunophenotypes and fusion transcripts (P>0.05) Table 2.

DISCUSSION

Association of *FHIT* gene has been widely witnessed with a wide range of tumor-suppressive properties like down regulation of oncogenic activity, apoptosis, invasion, and metastasis[18]. Any change that proceeds in fhit protein expression results in a number of changes at molecular level and causes alterations in its functional activity. Such changes ultimately lead to different kinds of diseases including tumorigenesis [9]. Expression of fhit protein has been detected in both normal and malignant human tissues. Various reports have observed that low *FHIT* expression has a significant association with the stage of many cancers. Reports are also available regarding the *FHIT* gene hypermethylation as an important contributor in causation of different malignancies [20].

In the present study, we observed a higher ALL risk among males and subject younger in age. As a main characteristic of ALL, maximum subjects were having a substantially higher WBC count and with precursor B ALL immunophenotype. Methylated promoter DNA sequences exhibited that methylation is a frequent event in the process of childhood ALL development as observed in our study also. Maximum numbers of subjects' were with negative fusion transcript and showed hypermethylation. The promoter methylation was higher in males, in subject's≥18 years of age, patients with lower platelet count, higher WBC count.

The higher number of male subject with hypermethylation in current study is in compliance to earlier reported results . The possible reason for higher risk among males could be higher number of males in study group. Similarly, the higher incidence of ALL among subjects with younger age in current study is due to more cell proliferation (the main characteristic feature of ALL cells) in the initial stages of life as compared to older age .

The higher WBC count associated with predominant precursor B-ALL immunophenotype in our study is in agreement with earlier reports. The higher number of WBCs though does not always specify ALL, but it also indicates the underlying problems due to ALL like infection, stress, inflammation, trauma etc. The higher percentage of secondary precursor B-ALL immunophenotype in our is in compliance to already published reports which have shown precursor B-ALL as a characteristic feature of lymphoblast proliferation arrested at an early stage of B-cell maturation. ALL cases with secondary precursor B-ALL had an inferior survival; the differences were likely attributed to the poor-risk cytogenetic abnormalities in these patients as a result of cytotoxic exposure. Nonetheless our data indicate that secondary precursor B-ALL can be stratified by the current precursor B-ALL risk stratification system similar to de novo precursor B-ALL, in which cytogenetics appears to be the most important factor in predicting survival.

We observed 80% of the subjects with *FHIT* hyper methylated gene. Such observation have also been reported earlier in different parts of the world but results were inconsistent 4. This kind of epigenetic modification can have significant effect on chromosomal structure and transcriptional activity particularly among known candidate tumor suppressor genes and hence result in genetic aberrations and tumorigenesis including leukemia's 6. Earlier reports have shown that methylation-associated silencing inactivates certain tumor suppressor genes as effectively as mutations and thus could act as cancer-predisposing hits 7. Like our study that found a majority of ALL cases with hypermethylated FHIT promoter sequence, earlier reports have furnished ample evidence that DNA methylation is the most frequent phenomena that occurs in the event of ALL28-[31]. Eventually it has been substantiated that hypermethylation of a series of genes

may be implicated in the relapse of pediatric ALL[32].The frequency of such process, the type of gene involvement, and the wide range of malignancies shown to harbor methylated promoter CpG islands, reflect the key importance of epigenetic mechanisms in cancer initiation and progression of malignancies. As already reported, there is tissue specificity of hyper methylated genes in different malignancies, therefore, *FHIT* gene hypermethylation present substantially higher in study ALL cases could have an important prognostic importance among ALL patients.

The limitations of the study include lack of survival analysis with respect to *FHIT* methylation status and assessment of fhit protein expression to analyze concordance between the methylation and expression

CONCLUSION

We conclude that *FHIT* gene hypermethylation is an important genetic event that is highly implicated in ALL cases and was found as an important event in the ALL pathogenesis. The results further need to be investigated in large cohort of samples in different populations to substantiate the role of *FHIT* gene in ALL.

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Ethical approval: The procedures done that involved human participants were performed in agreement with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The ethical approval was acquired from Institutional Ethical Committee (IEC/SKIMS protocol: 99/2016).

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