The correlation between SMG1 promoter methylation and its expression in acute lymphoblastic leukemia patient

Hoda Pourkarim1, Mehdi Azad2, Mohammad Taghi Haghi Ashtiani3, Samaneh Keshavarz4, Fatemeh Nadali1*

1 Department of Hematology and Blood Banking, Faculty of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran
2 Department of Hematology and Blood Banking, Faculty of Allied Medical Sciences, Qazvin University of Medical Sciences, Qazvin, Iran
3 Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
4 Department of Hematology and Blood Banking, Faculty of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Background: Acute lymphoblastic leukemia (ALL) comprises a heterogeneous group of disorders which originate from various important genetic lesions in B and T progenitor cells, including mutations that lead to stage-specific developmental arrest and those that impart the capacity for unlimited self-renewal, resulting in clonal expansion of immature progenitor cells. Acute lymphoblastic leukaemia occurs in both children and adults but its incidence peaks between 2 and 5 years of age. Causation is multifactorial and exogenous or endogenous exposures, genetic susceptibility, and chance have roles. Survival in pediatric acute lymphoblastic leukaemia has improved to roughly 90% in trials with risk stratification by biological features of leukaemic cells and response to treatment, treatment modification based on patients' pharmacodynamics and pharmacogenomics, and improved supportive. The promoter methylation pattern of DNA in cancer cells is different with the normal cells. Suppressor with morphogenetic effect on genitalia family member (SMG1) belongs to a family of phosphoinositide 3-kinase-related kinases and is the main kinase involved in nonsense-mediated mRNA decay.

Materials and Methods: This study was performed to investigate the correlation between SMG1 promoter methylation and its expression levels in acute lymphoblastic leukemia using methylation specific PCR (MSP). Our patients and control samples were collected from Children's Medical Center of Tehran and Imam Khomeini hospital of Tehran. To confirm the MSP results, we used Quantitative Real time-PCR (qRT-PCR) to measure the expression level of mRNA to find out if there is any relation between pattern of methylation and expression.

Results: After performing MSP, we found that SMG1 promoter was hypermethylated. Hyper methylation of SMG1 was detected in 67/74% (21/31) of ALL samples compared to control group. SMG1 mRNA expression was down-regulated 2.74 fold compared to control group.

Conclusion: The aim of this study was to investigate the effect of methylation pattern on gene expression. Our findings suggest that SMG1 acts as a functional tumor suppressor gene which was down-regulated by CpG islands hypermethylation in ALL patients. It was shown that the methylation of SMG1 was occurred in the 67/74% of samples.

Keywords: Acute lymphoblastic leukemia (ALL). SMG1, DNA Methylation
**Introduction**

Acute lymphoblastic leukemia (ALL) is the most common cancer in children and occurs with high frequency in childhood and is associated with high mortality in adults. So, the detection of residual leukemic cells (minimal residual disease, MRD) is the most important prognostic factor to identify high risk patients(1).

Patients are mainly children; roughly 60% of cases occur in people aged younger than 20 years (1),(2, 3) and Survival in childhood acute lymphoblastic leukemia is approaching 90% (3) but treatment in infants (i.e. children younger than 12 months) and adults needs improvement (4, 5).

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive blood malignancy that arises from the malignant transformation of T-cell progenitors and is predominantly diagnosed in children and adolescents where it accounts for 10–15% of ALL cases with a second peak of recurrence in aged adults (25% of adult ALL cases) (6). During this maturation process, cooperation between a variety of oncogenes and tumor suppressors can drive immature thymocytes into uncontrolled clonal expansion and cause T-cell acute lymphoblastic leukemia (T-ALL). Recent genetic studies have identified recurrent somatic alterations in genes involved in DNA methylation and post-translational histone modifications in T-ALL, suggesting that epigenetic homeostasis is critically required in restraining tumor development in the T-cell lineage.

Recent studies have proved that, not only the successive accumulation of genetic alterations in oncogenes and tumor suppressor genes, but also the epigenetic alterations contributed to carcinogenesis(7).

Epigenetic is a mechanism of gene expression regulation that does not alter gene sequence (8, 9). The most commonly occurring epigenetic event is DNA methylation at cytosine that precedes guanine in the DNA sequence, the CpG dinucleotides. The addition of a methyl group at the carbon 5 position of the cytosine ring is catalyzed by DNA methyltransferases (DNMTs) (8, 10). Due to the high mutagenic potential of 5-methylcytosine, CpG dinucleotides are irregularly distributed along human genome. There are CpG-rich regions—the CpG islands—in the regulatory region of many genes, namely tumor suppressor genes. In general, CpG islands are normally unmethylated, but repetitive genomic sequences and introns are hypermethylated (10, 11).

SMG1 is a common tumor suppressor gene. SMG1 is a PI3K-related kinase (PIKK) associated with multiple cellular functions, including DNA damage responses, telomere maintenance, and nonsense-mediated mRNA decay (NMD). NMD degrades transcripts that harbor premature termination codons (PTCs) as a result of events such as mutation or alternative splicing (AS). Recognition of PTCs during NMD requires the action of the upstream frameshift protein (Upf1) which must be phosphorylated at first by SMG1. However, the physiological function of mammalian SMG1 is not known (12).

**Methods**

**Patients and WBC isolation.** A total of 31 subjects including leukemia patients (10 men and 21 women), with a mean age of 4/6 years (range 1-12) and 5 healthy controls were participated in this study. Before beginning, we collected consent from their parents. The clinical data for leukemia patients are shown in Table1.

Peripheral blood (5ml) samples were collected and transferred to laboratory. After that, the RNA and DNA extraction process was done. Mononuclear cells of drawn samples including leukemic blast cells were isolated by concentration gradient sedimentation using

<p>| Table 1: Clinical data for leukemia patients |</p>
<table>
<thead>
<tr>
<th>Parameters</th>
<th>ALL patients (Mean ± Standard Deviation)</th>
</tr>
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<tbody>
<tr>
<td>age</td>
<td>4.6</td>
</tr>
<tr>
<td>WBC count</td>
<td>11.7</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>1.37</td>
</tr>
<tr>
<td>Platelet count</td>
<td>61.90</td>
</tr>
</tbody>
</table>
Ficoll-hypaque (GE Healthcare, Waukesha, WI, USA). We extract the DNA with GENE ALL kit (qiagene) according to the manufacturer’s procedure, then they treated with bisulfite by using the FAST Epi Tec Bisulfite Kit (Qiagene59824) according to the manufacturer’s instructions.

**Total RNA extraction and cDNA synthesis.** Total RNA were extracted from fresh peripheral blood of ALL patients using TRIZol reagent (Invitrogen, Cat num. 15596-026). 6 µL of purifies RNA were used for the quality control purposes and the rest were stored at -70°C or used immediately for cDNA synthesis. To check the quantity and quality of RNA, we used both nano-drop device (Thermoscientific) and electrophoresis on 1% formaldehyde agarose gels.

**MSP.** We used MSP for investigating the methylation pattern of SMG1 promoter. For this reason, we used 2 pairs of primers specified for checking the methylated and unmethylated residue.

**Primer design for qRT-PCR.** All the mRNA transcript sequences of the SMG1 and GAPDH (as reference gene) genes were downloaded from NCBI (www.ncbi.nlm.nih.gov) and Ensemble (www.Ensemble.org) databases. We performed a multiple sequence alignment using MEGA6 software to select a conserved region in order to amplify all the possible variants of the target genes. Then, the conserved regions were inserted into Oligo7 software for qRT-PCR primer design.

After designing, the primers were analyzed by NCBI BLAST. The BLAST results also showed high specific primers for all target genes which amplify almost all of the important mRNA variants. The primer pairs information for the target gene and reference gene are mentioned in the following table (Table3).

**Real Time PCR.** In order to investigate whether the promoter hypermethylation of SMG1 gene results in the reduced SMG1 expression or not, we performed qRT-PCR to detect the SMG1 expression in ALL patients and control group. We used ABI7500 for our experiment. Melting curve analysis was performed for both genes at the end of each run. Because of their efficiency, difference was less than 3%, so analysis of the expression result was done with 2−ΔΔCt method that shows the expression changes. Specificity of experiment was investigated by melt curve and electrophoresis. Real time PCR was carried out in volume of 20µl containing: 10µl qPCR master mix (Amplicone), 2µL of cDNA and 1µl mixed forward and reverse primers and 7 µl ddH2O. GAPDH was used as housekeeping gene.

**Statistical analysis.** For all calculations, SPSS (Statistical Package for Social Sciences) software version 20 was employed. The Mann-Whitney U test was used to compare SMG1 expression level in methylated and unmethylated group. A p-value of less than 0.05 was regarded as statistically significant.

**Results**

All samples were used in real time PCR. The

| Table 2: Primer sequence (M and U stand for methylated and unmethylated, respectively) |
|---------------------------------|------------------|
| SMG1 M-forward primer sequence | 5'-GGGTACGTGAATTTAAGGGTAC-3' |
| SMG1 M-reverse primer sequence | 5'-AACAAAAATCTCCACTACTACGAC-3' |
| SMG1 U-forward primer sequence | 5'-GGGTATGGAATTTAAGGGTATGT-3' |
| SMG1 U-reverse primer sequence | 5'-AACAAAAATCTCCACTACTACAAC-3' |

| Table 3: Real time PCR primer information for the target gene |
|-----------------|-----------------|------------------|
| Gene | type | Sequence | Product size |
| SMG1 | F | GTG GAG AGT TAC GCA GTC TT | 282 |
| SMG1 | R | CGC ATA ATG TGT AAA ACCTGCTC | 282 |
| GAPDH | F | GCTCTCTGCTCCTCTGTTTC | 114 |
| GAPDH | R | CGACCAAATCCGTTGACTCC | 114 |
ratio of SMG1 to GAPDH transcripts was reduced 2.74 folds in the methylated group compared to that in the control group. These results strongly indicated that hypermethylation in the promoter region of SMG1 gene was responsible for the lower expression of SMG1 in ALL.

SMG1 promoter is completely methylated in 21 patients (67.7%) and completely unmethylated in 9 patients (29.1 %). None of control individuals showed methylation in SMG1 gene.

In ALL patients, hypermethylation frequency of SMG1 was 67.7% (22 out of 31 patients).

**Discussion**

While cancer has been long recognized as a disease of the genome, the importance of epigenetic mechanisms in neoplasia was acknowledged more recently. The most active epigenetic marks are DNA methylation and histone protein modifications and they are involved in basic biological phenomena in every cell. Their role in tumorigenesis is stressed by recent unbiased large-scale studies providing evidence that several epigenetic modifiers are recurrently mutated or frequently dysregulated in multiple cancers. The interest in epigenetic marks is especially due to the fact that they are potentially reversible and thus druggable (13).

Epigenetic and its different mechanism are an important part of the gene expression regulation. Epigenetic changes lead cause to distinctive changes in gene expression that occur without any change in the gene sequence. Epigenetic is study of chromatin biochemical changes consists of DNA methylation and various histone modifications and changes in non-coding micro-RNA that each of these mechanisms can affect gene expression and gene silencing without sequence changing.

Tumor suppressor genes silencing, is a common phenomenon in cancer. In fact, methylation is one of the most common mechanisms of gene silencing in most of suppressor genes. DNA methylation is an enzyme depends on chemical modification that alters structure of DNA bases.

In vertebrates, methylation occurs in cytosine in the 5' with a guanine. Methyl cytosine bases are approximately constituted of 1% of the human genome that 70 to 80 percent of which located in CpG islands. Most of the CpG islands (94%) are remain unmethylated in normal cells and gene expression occurs based on the need of cell (14).

In present study, we investigated the methylation pattern of SMG1 promoter in 31 ALL patients at diagnosis stage and before treatment and in the 5 blood samples of normal people. Our study is the second research about the SMG1 methylation in hematological malignancies. Before this, there is only one study which investigate the methylation of SMG1 promoter in ALL (15) and is the first study in which we found that the SMG1 mRNA expression was downregulated because its promoter was hypermethylated in ALL.

The first study about SMG1 in leukemia is about AML which was done in 2014 and studied the methylation pattern and its expression. Their results showed that SMG1 was hypermethylated in AML and it was down-regulated. Our results showed that 21 out of 31 ALL patients that are in the first stage of ALL diagnosis, have methylated SMG1, in other words, in 67.7% of ALL samples before diagnosing, hypermethylation was occurred whereas no methylation was seen in the normal group. In addition, we measured the expression levels of SMG1 mRNA in patients with real time PCR. The results indicate that expression level in samples with methylation is 2.74-fold lowered compared to normal control samples.

The most direct mechanism by which DNA methylation can interfere with transcription is to prevent the binding of basal transcriptional machinery or ubiquitous transcription factors that require contact with cytosine in the major groove of the double helix. From another point of view CpG island chromatin is enriched in hyperacetylated histones and deficient in linker histones. These are essential features of transcriptionally competent chromatin templates. In contrast, chromatin assembled on artificially methylated DNA becomes associated with hypoacetylated histones, refractory to nuclease or restriction endonuclease digestion and transcriptionally silent (16).

In another study which was done in 2016 by Patricia Rebeiro, Alexander James, they investigated the role of SMG1 expression in B cell lymphoproliferative disease.
Loss of SMG1 leads to the development of B cell lymphoproliferative disease in mice. However, this has not been studied in humans. The aim of their project is to correlate SMG1 expression in human B cell lymphoproliferative disease with clinical characteristics, histology, karyotype, response to therapy and prognosis. This is done by immunoblotting on purified clonal B cells in human blood and tissue microarray on human lymphoma. Preliminary results show that there is significant heterogeneity in SMG1 expression in chronic lymphocytic leukemia and mantle cell lymphoma. This study highlights a potential important pathway in the pathogenesis and treatment of B cell lymphoproliferative disease (17).

In other studies, the tumor suppressor role of SMG1 and the alternation of its expression level were studied in other cancers. For example, in a study conducted in 2010, SMG1 role as a NMD physiological factor was studied. NMD in addition to its role in removing of the mRNA with premature stop codon, SMG1 has an important role in the regulation of many RNA-binding transcription factors, too. In this study with SMG1 nude mouse model, they demonstrated that it is necessary for embryonic development and in its absence, mRNAs with PTCs will remain in the cell (18).

In a study of hepatocellular carcinoma that was conducted in 2015, it has been concluded that the AZD5363 inhibits AKT pathway downstream molecules and mTOR and SMG1 activation dependon the cell type involved in the cancer (19).

In another study in 2013, it was reported that SMG1 regulates the G1/S check point cell cycle due to radiation response by phosphorylation of p53. It causes its stability and activation. In fact, cell cycle and tumor growth regulation are done in p53 dependent pathway. SMG1 inhibits CDK2 in response to DNA damage.

In another study that was done in 2011 by HH cheung et al, the regulatory role of SMG1 and NIK in apoptosis induced by SMCs (Smac mimetic compounds) has been investigated. SMCs are experimental small molecules that induce tumor necrosis factor alpha. They indicated that such protein kinases like NIK and SMG1 have an important role in protecting cancer cells in induced Smac-mediated TNF-α apoptosis. In fact, they found a new role for SMG1 and NIK as SMCs -mediated TNF-α induced inhibitors (20).

**Conclusion**

Taken together, our findings show that SMG1 as a tumor suppressor gene was down-regulated by CpG island hypermethylation in ALL patient. The methylation of SMG1 was occurred in the 67/74% of samples. The SMG1 mRNA expression level of methylated samples is 2.74 fold lower than the normal group. It is recommended to conduct more studies to determine the role of hypermethylation of SMG1 in the pathogenesis of ALL or in ALL subtypes and other hematologic malignancies and observe an association between methylation of SMG1 and clinical findings in ALL patients such as age, sex, WBC, platelet count and complete remission after induction therapy .Moreover investigations of other regulation mechanism of SMG1 in ALL are needed. We also suggest to use other methylation technics to investigate the methylation pattern and also study the ALL in cell lines.

**Conflicts of Interest**

The authors declare that there is no conflict of interest in this study.

**Acknowledgment**

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