Original Article

# Methylation and Expression Status of The CpG-Island of *SMG1* Promoter in Acute Myeloid Leukemia: A Follow-Up Study in Patients

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Abstract

**Objective:** Aberrant alterations in DNA methylation are known as one of the hallmarks of oncogenesis and play a vital role in the progression of acute myeloid leukemia (AML). *SMG1* is a member of the Phosphoinositide 3-kinases family, acting as a tumor suppressor gene. The aim of this study was the evaluation of the expression level and methylation status of *SMG1* in AML.

**Materials and Methods:** In this follow-up study on AML patients admitted to Shariati Hospital, Tehran, Iran, the methylation status of *SMG1* [performed by methylation-specific polymerase chain reaction (PCR)] and its expression level (performed by qRT-PCR) were evaluated in three phases: newly diagnosed, under treatment and complete remission. The correlation of the methylation status of *SMG1*, its expression level, and clinical/paraclinical data was analyzed by SPSS ver.25.

**Results:** This study on 18 patients and five control individuals showed that the CpG-islands of the *SMG1* promoter in newly diagnosed cases is hypomethylated compared to the normal group (P=0.002) The fold change of *SMG1* expression levels in new cases is 0.464 ± 0.468, while the fold change of *SMG1* expression levels in under-treatment and in-remission patients is 0.973 ± 1.159 and 0.685 ± 0.885, respectively. In under-treatment patients, white blood cell (WBC) count decreases 114176.36 cell/μl with each unit of increase in fold change of SMG1 (P<0.0001), and Hb unit increases 2.062 g/dl with each unit of increase in fold change (P<0.0001) Also, in the remission phase, the Hb unit increases 1.395 g/dl with each unit increase in fold change (P=0.019).

**Conclusion:** The robust results of our study suggest that the methylation and expression of SMG1 have a high impact on the pathogenesis of AML. Also, the methylation and expression of *SMG1* can play a prognostic role in AML.

***Keywords:*** Acute Myeloid Leukemia, DNA Methylation, Follow-Up Studies, SMG1

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## Introduction

Acute myeloid leukemia (AML), as a hematopoietic malignancy, is the most common form of acute leukemia in adults and involves abnormal proliferation and differentiation of hematopoietic stem cell colonies (1). AML presents with more than 20,000 new cases per year in the United States alone. The prevalence of AML is three to five individuals per 100,000. The distinct cellular feature in AML is abnormal myeloid cell development and neoplastic proliferation in the bone marrow (2). Also, some cytogenetic abnormalities lead to complications in diagnosis, prognosis, and treatment (3), making necessary to investigate novel approaches for this type of leukemia.

DNA methylation is a gene expression regulatory mechanism occurring in GC-rich sites of the genome. Hypermethylation of the CpG-islands of tumor suppressor genes leads to tumorigenesis. Also, hypomethylation in the CpG-islands of proto-oncogenes is one of the events causing cancer. Aberrant DNA methylation alterations are known as one of the hallmarks of oncogenesis and play a vital role in the progression of AML (4, 5).

*SMG1* (Suppressor with morphogenetic effect on genitalia family member 1) is considered a tumor suppressor gene. Dysregulation of *SMG1* leads to tumorigenesis. *SMG1* is a member of the Phosphoinositide 3-kinases family, involved in nonsense-mediated decay (NMD) (6). Also, *SMG1* participates in initiating DNA damage responses, telomere retention, oxidative/hypoxic stress responses, and stress granule formation. *SMG1* is required for the G1/S checkpoint site maximum activity for ionizing radiation exposure or during oxidative stress. Complete absence of *Smg1* expression during the early stages of mouse fetal development causes the fetus to die.

Also, the absence of a single allele of *SMG1* increases the risk of cancer, especially lung adenocarcinoma and lymphoma. *SMG1* deficiency causes high levels of basal inflammation and oxidative damage of tissue in the pre-cancerous stage, which may indicate the role of this cascade in carcinogenesis (7, 8)

Regarding the lack of theoretical and experimental knowledge about AML and the impact of methylation in this leukemia, we investigated the *SMG1* CpG-island methylation patterns in AML patients and its correlation with the *SMG1* expression level to introduce a potential hallmark in hematopoietic malignancy. We also investigated the effect of *SMG1* expression on paraclinical indexes as the therapeutic outcome.

## Material and Methods

### Sampling

In this follow-up study, 18 patients with AML who had been admitted to Shariati Hospital, Tehran, Iran, were studied, whose AML had been confirmed based on laboratory tests. The patients were separated into three groups: new cases, receiving medications, and in remission. Also, five healthy individuals were considered as control. The signed informed consent was obtained from all participants. This study was approved by the ethics committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1397.198). All samples were collected in heparin-lithium CBC tubes. All patients received a regular therapeutic regime for AML based on FDA protocol (Cytarabine for seven days and Anthracycline drugs such as Daunorubicin (Daunomycin) or Idarubicin three days).

### DNA extraction and bisulfite treatment

The DNA was extracted using GeneAll kit (GeneAll, South Korea), as per the manufacturer's protocol. The bisulfite treatment was performed to replace unmethylated cytosine residues with uracil, using EpiTect Fast DNA Bisulfite Kit (Qiagen, USA), following the manufacturer's protocol.

### Methylation-specific PCR for the *SMG1* CpG-island

Methylation-specific PCR (MSP) was conducted for the amplification of bisulfite-treated DNA. For this aim, 10 µl of TEMPase Hot Start 2x Master Mix BLUE (Ampliqon, Denmark), 1 µl of each set of reverse and forward primers of methylated and unmethylated sets (Table 1), 1 µl of bisulfite-treated DNA template were used and adjusted to the final volume of 20 µl using $ddH_2O$. The thermal cycling of MSP was performed using ABI Applied Biosystems™ (Thermofisher, USA) as follows: 10 minutes in 95˚C for pre-denaturation, and 30 cycles including 15 seconds at 94˚C for denaturation, 30 seconds at 53˚C for denaturation, and 15 seconds at 72˚C for the extension, per cycle. Also, the amplicons were incubated for 10 minutes at 72˚C for a final extension. For detection of the methylation status of the *SMG1* promoter in AML patients and healthy individuals, the MSP products were loaded on 1% agarose electrophoresis gel. Positive and negative controls for methylation were used to verify the accuracy of the MSP. EpiTect Control DNA Bisulfite converted (Qiagen, USA) was used for MSP control ( [Methylated control (lot No: 157047896) and unmethylated control (lot No: 157045952)].

### RNA extraction and cDNA synthesis

The total RNA of samples was extracted using the GeneAll RNA extraction kit (GeneAll, South Korea), as per the manufacturer's protocol. The reverse transcription of extracted RNA samples was performed using Thermo Scientific RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA), following the manufacturer's protocol.

### *SMG1* expression level

Real-time PCR was used to evaluate *SMG1* expression level in patients and healthy individuals, using 7.5 µl of RealQ Plus 2x Master Mix Green Without ROX™ (Ampliqon, Denmark), 0.5 µl of each primer (forward and reverse), and 1 µl of cDNA, which was adjusted using $ddH_2O$. Real-time PCR stages were conducted using ABI Applied Biosystems™ (Thermofisher, USA) as bellow: 15 minutes at 95˚C for pre-denaturation, and 19 seconds at 95˚C for denaturation, 19 seconds at 61.5˚C for denaturation and extension, per cycle. The Rotor-Gene device (Qiagen, USA) was used to perform thermal processes. Also, the *GAPDH* gene was used as the internal control gene. The sequences of forward and reverse primers of *SMG1* and *GAPDH* genes are given in Table 1.

### Hematopoietic laboratory indexes

White blood cells (WBC, cells/µl), red blood cells (RBC, cells/µl), platelets (Plt, cells/µl), and hemoglobin (Hb, g/dl) were measured using Sysmex cell counter (Sysmex Corporation, Japan). All parameters were evaluated in control individuals and all studied phases in patients.

### Statistical analysis

The multiple linear model and ordinal logistic regression were used to identify the correlations. All statistical analysis was performed by SPSS software, version 25 (IBM, USA). The significant level was considered as 5%. Also, Ct values of real-time PCR results were calculated using the REST software.

## Results

### Sampling characteristics

Out of a total of 18 patients (seven males and 11 females, aged 15 to 67) admitted to Shariati Hospital, Tehran, Iran, nine patients were monitored for methylation status of the *SMG1* promoter in three phases of the disease (newly

diagnosed/under treatment/remission). Four patients participated in two phases (under treatment/ remission), and five patients were involved in only one phase (newly diagnosed cases).

### Methylation status of the CpG-island of the *SMG1* promoter in patients in different phases of AML

While all control individuals show the hemi-methylated status in their CpG-islands of *SMG1*, the results of Ordinal Logistic Regression analysis show that the methylation status of the CpG-islands of the *SMG1* in newly diagnosed cases is significantly hypomethylated compared to the control group (P=0.002). Also, there was no significant difference in the methylation status of the CpG-islands of *SMG1* between the patients in the under-treatment phase and remission phase with the control group (P=0.236 and P=1.000, respectively, Fig.1). The demographic data of the methylation pattern frequency in participants are reported in Table 2.

### *SMG1* expression level in different phases of acute myeloid leukemia

The *Pfaffl* statistics showed that the fold change of *SMG1* expression levels in new cases is 0.464 ± 0.468, while *SMG1* expression levels in the under-treatment and in-remission patients are 0.973 ± 1.159 and 0.685 ± 0.885, respectively. Therefore, the expression level of *SMG1* in new cases and in-remission patients are reduced compared to the control group.

Multiple linear models showed that in the remission phase, the fold changes are significantly different between patients with methylated and unmethylated Promoters (P=0.001). Also, in the remission phase, the fold changes are significantly different between hemi-methylated and unmethylated patients (P=0.002, Table 3).

**Table 1:** Methylated and un-methylated primers for MSP of SMG1 CpG-islands and the primer sequences of *SMG1* and *GADPH* (internal control) for evaluating the expression level of *SMG1* by real time polymerase chain reaction

| Methylation state of primers | Length (bp) | Primer sequence (5′-3′) |
|---|---|---|
| Methylated primer | 22 | F: GCGTACGTGAATTTAAGGGTAC |
| | 25 | R: AACAAAAAATCTCCACTACTACGAC |
| UnMethylated primer | 25 | F: GGTGTATGTGAATTTAAGGGTATGT |
| | 25 | R: AACAAAAAATCTCCACTACTACAAC |
| *SMG1* | 20 | F: GTGGAGAGTTACGCAGTCTT |
| | 23 | R: CGCATAATGTGTAAAACCTGCTC |
| *GAPDH* | 20 | F: CAATGACCCCTTCATTGACC |
| | 20 | R: TGGAAGATGGTGATGGGATT |

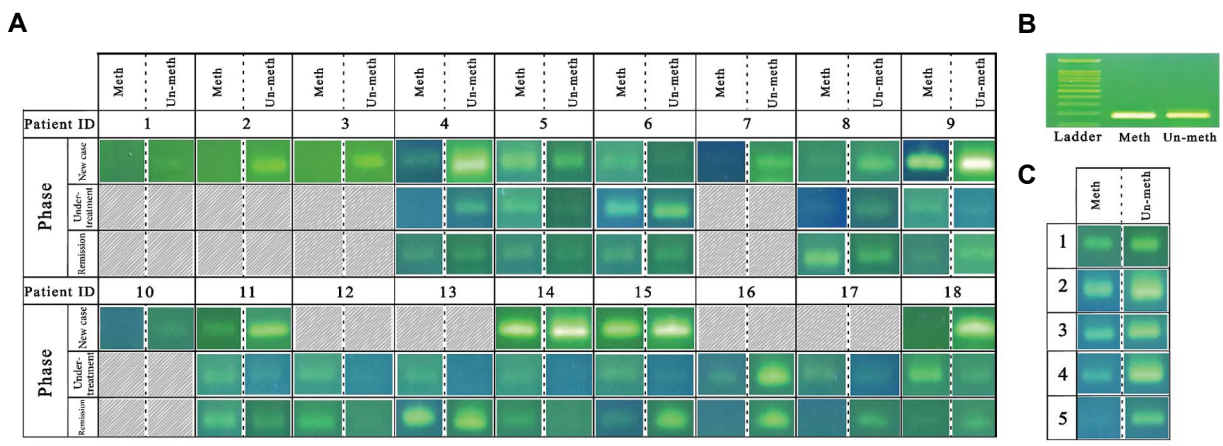


**Fig.1:** Methylation status of *SMG1* gene promoter in acute myeloid leukemia (AML) patients. **A.** AML patients were classified into three phases: new cases, under-treatments, and in-remissions. The methylation status of *SMG1* was evaluated by MSP, using methylated (Meth) and unmethylated (Un-meth) specific primers. The results were shown on 1.5% agar gel electrophoresis. **B.** Positive and negative control of MSP. **C.** MSP of the *SMG1* promoter in healthy individuals (control group). Most healthy individuals show hemimethylated status in the *SMG1* promoter.

**Table 2:** Demographic data of methylation status in different phases of AML

| Methylation status | | Phase | | | Total |
|---|---|---|---|---|---|
| | | New cases | Under treatment | Remission | |
| Un-methylated | Count (% within phase) | 6 (42.9) | 1 (7.7) | 2 (15.4) | 9 (22.5) |
| Hemi-methylated | Count (% within phase) | 8 (57.1) | 9 (69.2) | 9 (69.2) | 26 (65.0) |
| Methylated | Count (% within phase) | 0 (0.0) | 3 (23.1) | 2 (15.4) | 5 (12.5) |
| Total | | 14 (100) | 13 (100) | 13 (100) | 30 (100) |

Data are presented as n (%). AML; Acute myeloid leukemia.

**Table 3:** Correlation of fold changes in different phases and methylation statuses

| Phase | Methylation status | | Mean difference (95% Wald confidence interval for difference) | P value |
|---|---|---|---|---|
| Remission | Methylated | Hemi-methylated | -.0778 (-0.2465, 0.0910) | 0.366 |
| | | Un-methylated | -2.1500[a] (-3.4739, -0.8261) | 0.001 |
| | Hemi-methylated | Un-methylated | -2.0722[a] (-3.9323, -0.7521) | 0.002 |
| Under- treatment | Methylated | Hemi-methylated | -.0750 (1.2334, 1.0834) | 0.899 |
| | | Un-methylated | 0.3500 (-0.4122, 1.1122) | 0.368 |
| | Hemi-methylated | Un-methylated | 0.4250 (-0.4472, 1.2972) | 0.340 |
| New cases | Hemi-methylated | Un-methylated | 0.1125 (-0.3256, 0.5506) | 0.615 |

## Correlation of *SMG1* expression and paraclinical indexes

The analysis of the interaction of phase and methylation status shows that no one of the paraclinical indexes is significantly different (Table 4). Also, WBC, RBC, Plt, and Hb are all significantly different in different phases (P<0.001, for all) While WBC and Plt counts are significantly different in different methylation statuses (P=0.018 and P=0.029, respectively), Hb and RBC are not different in patients with different methylation status.

The results of the generalized estimating equation (GEE) statistical test in patients who participated in three phases of this trial show that in under-treatment patients, WBC count decreases 114176.36 cell/µl on overage with each unit of increase in fold change [P<0.001, 95% confidence interval (CI): (-177285.38, -51067.34)]. Also, in the remission phase, WBC count averagely decreases 115229.26 cell/µl with each unit of increase in fold change [P<0.001, 95% CI: (-178497.21, -51961.31)].

In under-treatment patients, Hb unit increases 2.062 g/dl with each unit of increase in fold change [P<0.001, 95% CI: (0.930, 3.195)]. Also, in the in-remission phase, Hb unit increases 1.395 g/dl with each unit of increase in fold change [P=0.019, 95% CI: (0.233, 2.558)].

Regarding the Plt count in under-treatment patients, the Plt count increases 36637.75 cell/µl with each unit of increase in fold change [P=0.012, 95% CI: (7999.16, 65276.36)]. There were no significant correlations between other indexes/phases and fold change of the *SMG1* gene (Table 5).

**Table 4:** Correlation of laboratory indexes in different phases and methylation status

| Laboratory indexes | Phase | | Methylation status | | Interaction of phase and methylation status | |
|---|---|---|---|---|---|---|
| | Wald chi-square (df=2) | P value | Wald chi-square (df=2) | P value | Wald chi-square (df=3) | P value |
| WBC (/µL) | 25.961 | <0.001 | 8.042 | 0.018 | 2.509 | 0.474 |
| RBC ($10^6$/µL) | 15.223 | <0.001 | 0.474 | 0.789 | 0.904 | 0.824 |
| Hemoglobin (g/dl) | 15.898 | <0.001 | 0.941 | 0.625 | 1.049 | 0.789 |
| Platelet (/µL) | 26.491 | <0.001 | 7.051 | 0.029 | 2.807 | 0.422 |
| Fold change | 15.478 | <0.001 | 4.718 | 0.095 | 4.718 | <0.001 |

WBC; White blood cell, RBC; Red blood cell, and df; Degree of freedom.

**Table 5:** Correlation of *SMG1* fold change and paraclinical indexes

| Indexes | | Under-treatment | Remission |
|---|---|---|---|
| WBC (cell/µl) | B (95% CI) | -114176.356 (-177285.376, -51067.337)* | -115229.26 (-178497.29, -51961.31) |
| | P value | <0.0001 | <0.0001 |
| RBC ($10^6$ cell/µl) | B (95% CI) | 0.552 (-0.061, 1.166) | 0.356 (-0.248, 0.960) |
| | P value | 0.078 | 0.248 |
| Hemoglobin (cell/µl) | B (95% CI) | 2.062 (0.930, 3.195) | 1.395 (0.233, 2.558) |
| | P value | <0.0001 | 0.019 |
| Platelet (cell/µl) | B (95% CI) | 36637.750 (7999.164, 65276.335) | -28148.811 (-73289.437, 16991.816) |
| | P value | 0.012 | 0.222 |

WBC; White blood cells, RBC; Red blood cells, and *; Change in amount of paraclinical indexes with each unit of increase in fold change compared with new cases.

## Discussion

Aberrant DNA methylation is a critical etiology in leukemia. The relative methylation of the CpG-islands of the SMG1 promoter, as a tumor suppressor gene, is involved in the progression of various types of cancers. Our results showed that methylation of the CpG-islands of the SMG1 promoter changed through the phases (from diagnosis to complete remission). In this study, it was demonstrated that the hemimethylated status of *SMG1* is dominant in all groups (control and cases), but in new cases (patients who have not received medication), the methylation status of *SMG1* is hypomethylated compared to the control, under-treatment and remission groups. Also, the distribution of the unmethylated alleles of *SMG1* is detected more frequently in new cases than control, medication-receiving, and in-remission groups. These finding show two facts: first, in AML, the epigenomic anti-cancer mechanisms lead to less methylation in the *SMG1* promoter, which leads to stronger tumor-suppressive effects of this gene, and two, the methylation status will return to a normal state following remission. The second finding can be due to the medications or the physiologic response of the body.

Different studies established that the *SMG1* gene acts as a tumor suppressor gene involved in various cancers, especially hematopoietic malignancies. On the other hand, CpG-island methylation patterns play a critical role in enhancing or inducing gene expression. Different studies established the role of epigenetics, especially DNA methylation, in the progression of hematopoietic malignancies (4). In order to correct the aberrant DNA methylation pattern, there are some methylation-targeting drugs. i.e., hypomethylating agents (HMAs), which have been developed for leukemia, lymphoma, and myeloma. Following the last studies on the impact of aberrant DNA methylation in cancer, various technologies are developed for gene-specific methylation modifications, i.e., CRISPR-Cas9-mediated methylome modifiers (9, 10). Alongside

the progression in methylation modifier technologies, the investigations are held on finding more methylation-based therapeutic, diagnostic, and prognostic biomarkers (11). In this study, we tried to investigate the role of the CpG-islands methylation patterns of *SMG1* in AML progression and its status during follow-up of patients

Our results showed that in new cases, the fold change of *SMG1* expression levels is 0.464 ± 0.468, while the CpG-islands of *SMG1* were in hypomethylated status, compared to the control group. Therefore, in new cases, the regulation of *SMG1* expression is not affected by promoter methylation. Also, *SMG1* expression levels in under-treatment and in-remission patients are 0.973 ± 1.159 and 0.685 ± 0.885, respectively, and the CpG-islands of *SMG1* are partially methylated compared to the control group. Therefore, the *SMG1* expression level is regulated by methylation of its promoter when patients receive medications (under-treatment and in-remission patients). Also, the regulation of *SMG1* expression in not affected by promoter methylation in new cases, but in under-treatment and remission phases, the *SMG1* expression level and promoter methylation is close to the control group.

Regarding the role of *SMG1* methylation status in cancer, Gholipour et al. (12) utilized that the CpG-islands methylation pattern of *SMG1* is in hemimethylated status in multiple myeloma patients. Gubanova et al. (13) showed that the CpG-islands methylation pattern of *SMG1* is in the hypermethylated state in head and neck cancer patients, compared to healthy individuals. Pourkarim et al. (7) showed that the CpG-islands methylation pattern of *SMG1* is hypermethylated in acute lymphoblastic leukemia (ALL) patients. A study was conducted in 2019 by Ho et al. (14) to investigate the effect of *SMG1* and *ATM* on mice. In mice, complete loss of fetal *Smg1* is fatal, and loss of a single allele increases the growth rate of cancers, especially hematopoietic cancers and lung cancer. The data showed that the simultaneous decrease in *ATM* and *SMG1* expression increased the progression of hematopoietic cancer. The results of this study confirm the importance of our study on the potential effects of *SMG1* on the incidence of AML. In a 2019 study by Mai et al. (15), they showed that miR-18a expression is upregulated in nasopharyngeal carcinoma tissues and is positively correlated with tumor size and tumor-nodes-metastases stage. *SMG1* was identified as the target of miR-18a. The results confirmed that miR-18a plays its carcinogenic role by suppressing *SMG1*, reducing its expression and activating the mTOR pathway in nasopharyngeal carcinoma cells. The results of this study, which indicate the importance of *SMG1* in the incidence of cancer, validate our results to show *SMG1* as a vital factor in the development of AML. A 2014 study by Du et al. (16) was conducted to evaluate the function of *SMG1* in AML. The results showed that *SMG1* was hypermethylated in the promoter. It should be noted that in this study, the relationship between *SMG1* gene expression and patients' clinical symptoms was not discussed. On the other hand, expression and methylation in different phases of the disease (at diagnosis, under treatment, and remission) were not studied.

In this study, we showed that the expression of *SMG1* is correlated to the *SMG1* methylation pattern. In the under-treatment group, the unmethylated allele of *SMG1* is most prevalent, while the expression level of *SMG1* is lower compared to other studied groups. In the remission group, the methylated allele of *SMG1* is more prevalent than in new cases and control, but not the under-treatment group. Furthermore, the expression level of *SMG1* is lower compared to new cases and control groups, while *SMG1* is more highly expressed in the remission group compared to the under-treatment group. These patterns are also the same in the control and new cases group. Regarding our findings, different studies established that the expression of *SMG1* is under the control of the CpG-islands methylation patterns of this gene.

The investigation of the correlation of *SMG1* expression and laboratory indexes showed that in under-treatment and in-remission patients, WBC count was reduced with each unit of increase in the fold change of *SMG1*. Also, the increase in fold change is responsible for the rise in Plt and Hb of patients in the under-treatment phase. Therefore, high expression of *SMG1*, as a tumor suppressor gene, leads to a better outcome in the remission phase of AML patients, regarding the induction of Plt generation and hematopoiesis and WBC reduction count.

Based on our results, the expression level and methylation status of *SMG1* is varied in different phases of AML and control individuals. Also, the expression level of *SMG1* is correlated with outcome-related laboratory hallmarks. Therefore, *SMG1* can be a potential prognostic biomarker for AML patients, requiring more studies.

## Conclusion

This study followed-up the methylation status and gene expression of *SMG1* in AML. In new cases, the CpG-island of *SMG1* is hypomethylated compared to the control group. Also, there are different expression levels in different phases and methylation statuses, but the expression level of *SMG1* is not regulated by promoter DNA methylation in new cases. Finally, due to the correlation of the expression level of *SMG1* and laboratory indexes, it can be suggested that *SMG1* expression and methylation status can predict the outcome of chemotherapy. The low number of participants, the mortality of involved patients in the follow-up process, and trouble in accessing patients were limitations of our study.

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## Authors' Contributions

N.K.; Has participated in study design, data collection and assessments, and conducting the molecular experiments. M.H.A., Sh.P.D.; Advised the molecular experiments and qRT-PCR analysis. S.M.; Contributed to data collection and assessments. A.M.; Contributed to data collection and drafting the manuscript. A.A.; Contributed to the advanced statistical analysis and data assessments. M.A.; Contributed to the conception, design, and all experimental works, and supervised this study. All authors read and approved the final manuscript.

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