Original Article

Aberrant DNA Methylation Status and mRNA Expression Level of *SMG1* Gene in Chronic Myeloid Leukemia: A Case-Control Study

Tahereh Hojjatipour, M.Sc.[1], Mahsa Sohani, M.Sc.[1], Amirhosein Maali, M.Sc.[2, 3], Shahrbano Rostami, Ph.D.[4*], Mehdi Azad, Ph.D.[5*]

1. Department of Hematology and Blood Transfusion, Students Research Center, School of Allied Medicine, Tehran University of Medical Sciences, Tehran, Iran
2. Department of Immunology, Pasteur Institute of Iran, Tehran, Iran
3. Department of Medical Biotechnology, School of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
4. Hematologic Malignancies Research Center, Tehran University of Medical Sciences, Tehran, Iran
5. Department of Medical Laboratory Sciences, School of Paramedicine, Qazvin University of Medical Sciences, Qazvin, Iran

Corresponding Addresses: P.O.Box: 3419915315, Hematologic Malignancies Research Center, Tehran University of Medical Sciences, Tehran, Iran
P.O.Box: 1416634793, Department of Medical Laboratory Sciences, School of Paramedicine, Qazvin University of Medical Sciences, Qazvin, Iran
Emails: drostamy@yahoo.com, haematologicca@gmail.com

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Abstract

Objective: Chronic myeloid leukemia (CML) is a myeloproliferative malignancy with different stages. Aberrant epigenetic modifications, such as DNA methylation, have been introduced as a signature for diverse cancers which also plays a crucial role in CML pathogenesis and development. Suppressor with morphogenetic effect on genitalia (*SMG1*) gene recently has been brought to the spotlight as a potent tumor suppressor gene that can be suppressed by tumors for further progress. The present study aims to investigate *SMG1* status in CML patients.

Materials and Methods: In this case-control study, peripheral blood from 30 patients with different phases of CML [new case (N)=10, complete molecular remission (CMR)=10, blastic phase (BP)=10] and 10 healthy subjects were collected. Methylation status and expression level of *SMG1* gene promoter was assessed by methylation-specific polymerase chain reaction (MSP) and quantitative reverse-transcription PCR, respectively.

Results: MSP results of *SMG1* gene promotor in the new case group were methylated (60% methylated, 30% hemimethylated and 10% unmethylated). All CMR and control group patients were unmethylated in the *SMG1* gene promoter. In the BP group, methylated *SMG1* promoter was seen (50% of patients had a methylated status and 50% had hemimethylated status). In comparison with the healthy subjects, expression level of *SMG1* in the new case group was decreased ($P<0.01$); in the CMR group and BP-CML groups, it was increased ($P<0.05$). No significant correlation between patients' hematological features and *SMG1* methylation was seen.

Conclusion: Our results demonstrated that aberrant methylation of *SMG1* occurred in CML patients and it had a significant association with *SMG1* expression. *SMG1* gene promoter showed diverse methylated status and subsequent expression levels in different phases of CML. These findings suggested possible participation of *SMG1* suppression in the CML pathogenesis.

Keywords: Chronic Myeloid Leukemia, DNA Methylation, Gene Expression, *SMG1*

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative malignancy characterized by excessive myeloid clonal proliferation in hematopoietic tissues (1, 2). Clinically, CML is divided into three phases, including the chronic phase (CP), accelerated phase (AP) and blastic phase (BP) (3). CP, AP and BP are defined as less than 10%, more than 10% and more than 20% of myeloid blasts in the blood and bone marrow, respectively. CP is the prolonged phase of CML that might last several years; in contrast, BP is considered the terminal phase of CML. Almost all CML patients have acquired the Philadelphia chromosome (Ph) resulting from a reciprocal translocation between the long arm of chromosome 9 and the short arm of chromosome 22. The translocation results in the formation of the *BCR-ABL1* gene fusion whose oncoprotein product is assumed as the main responsible for CML pathogenesis (4). However, the initial mechanism involved in the pathogenic Ph formation is unclear (1). As well as the Ph chromosome, multiple cytogenetic abnormalities and epigenetic dysregulations are reported, especially in CML progressed phases, such as AP and BP (2).

Epigenetic modifications, including DNA methylation, histone modifications and microRNA expression, are inducible and reversible changes that play a crucial role in gene regulation and cell cycle control. DNA methylation occurs in CpG dinucleotides, which are highly located in gene promotors. DNA methyltransferase (*DNMT*) enzymes are responsible for the DNA methylation

process by adding a methyl (CH_3) group to the 5′ position of cytosines (5). DNA hypermethylation usually leads to gene silencing, while DNA hypomethylation results in gene expression. Alternatively, tumor cells, such as CML, recruit the upper mentioned capacity through desired gene expression or suppression for further development (1). For instance, hypermethylation of some tumor suppressor genes, including E-cadherin, glutathione peroxidase 3 (*GPX3*), death-associated protein kinase (*DAPK*), estrogen receptor (*ER*), *p15* and *p16* have been reported in CML (5-7). Transcription Factor AP-2 Alpha *(TFAP2A*) also functions as a tumor suppressor gene and plays a critical role in cancer cell sensitivity to chemotherapy. Significant hypermethylation of the *TFAP2A* gene is reported in the BP of CML (8, 9). Several significant transcription factors such as DNA-damage-inducible transcript 3 (*DDIT3*), Runt-related transcription factor 1 *(RUNX1)*, Signal transducer and activator of transcription 3 (*STAT3*) and stem cell leukemia (*SCL*) were also hypermethylated in CML patients, especially in the progressed phases (6, 10, 11). Since the related function of upper-mentioned genes are critical in DNA repair and cell cycle regulation, their aberrant silencing has a significant impact on CML development (12, 13). On the other hand, there is sufficient evidence on the side of DNA hypomethylation in CML. The overall DNA hypomethylation leading to genomic instability is assumed as one probable reason for CML blastic transformation. Furthermore, hypomethylation of tumor-associated antigens and some oncogenes such as preferentially expressed antigen of melanoma *(PRAME)* has been shown in CML (1, 14).

Suppressor with morphogenetic effect on genitalia (*SMG1*) is a member of the phosphoinositide 3-kinase-related kinases (PIKK) family. The primary role of *SMG1* is participation in the non-sense mediating mRNA decay (NMD) process to eliminate premature mRNAs (15). However, previous research have revealed tumor suppressor activity of *SMG1* in solid tumors and hematologic malignancies (16). Several studies demonstrated the hypermethylated *SMG1* promotor and subsequent downregulation in different cancers (17, 18). Specific epigenetic modifications pattern has been shown in different cancers. For example, distinct methylation patterns have been reported in different AML subclasses, which probably associated to the particular cytogenetic and molecular abnormality (19, 20). In this regard, although many aspects of epigenetic abnormality in CML have been investigated, to the best of our knowledge, there is no data regarding *SMG1* status in CML patients. The aim of this study was to investigate methylation status and expression level of the *SMG1* gene in CML patients and determine association of this result with different phases of CML.

Materials and Methods

Patients and samples

This case-control study was performed on 30 CML patients, including 10 N (newly diagnosed CML with chronic phase), 10 complete molecular response (CMR) patients and 10 BP (blastic phase of CML) patients, as well as 10 healthy control participants referred to Hematology, Oncology and Stem Cell Transplantation Research Center at Shariati Hospital, Tehran, Iran. CML diagnosis was confirmed genetically in all cases by detecting the *BCR/ABL1* gene fusion. All medical records of patients were collected. The Human Research Ethics Committee from Tehran University of Medical Sciences approved the current study (IR.TUMS.VCR.REC.1395.68). Written informed consent was taken from the all participants and those who refused to participate in study were substituted with new participants. Five milliliters of arterial blood were collected in the sterile condition via vacuum tubes containing K2-EDTA from all participants and transferred to the molecular laboratory.

DNA extraction and bisulfite modification

DNA was extracted using DNA extraction kit (Favorgen Biotech, Austria) as manufaturer's protocol. Yield and purity of DNA were determined using a NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA). Then, DNA was treated with sodium bisulfite using Fast EpiTect Kit (Qiagen, USA). The treated DNA was resuspended in water and stored at -80˚C until the next steps.

Methylation-specific polymerase chain reaction

The treated DNA was prepared for methylation-specific polymerase chain reaction (MS-PCR) to investigate methylation status of the *SMG1* gene. The target DNA for PCR amplification was a 178 base pair segment of the 16p12.3 band, which was included in the CpG island of the *SMG1* promoter. The methylated (M) and unmethylated (U) primers of *SMG1* were designed with MethPrimer software.

MS-PCR method was performed using Taq DNA Polymerase Master Mix 2x (Ampliqon, Denmark). The PCR amplification was performed on the Applied Biosystems Veriti thermal (Applied Biosystems, USA). Each PCR tube contained 0.5 µl of forwarding methylated/un-methylated primers, 0.5 µl of reverse methylated/un-methylated primers, 10 µl of PCR master mix (2x), 8.5 µl of ddH_2O and 1 µl of bisulfite-treated DNA. The PCR was performed under the following condition: an initial pre-denaturation at 94˚C for 4 minutes, 35 cycles of PCR stages, including 94˚C for 30 seconds (denaturation), 62˚C for 30 seconds (annealing) and 72˚C for 30 seconds (extension), followed by a final extension of 5 minutes at 72˚C.

The PCR products were electrophoresed in a 2.5% agarose gel stained with DNA Safe Stain (Sinaclon, Iran, EP5083). Furthermore, for each MSP reaction a commercial methylated DNA (Qiagen Inc., cat. No. 59695) and a commercial unmethylated DNA (Qiagen Inc., cat. No. 59695) were used as methylated and unmethylated primer control, respectively. Distilled water was used as a non-template control in each set of PCR reactions.

RNA isolation and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using the TRIzol® Reagent (Thermo Fisher, USA). Yield and purity of RNA were determined using a NanoDrop spectrophotometer at 260/280 nm. cDNA was synthesized using the cDNA synthesis kit (Takara Bio Inc, Japan). The primers of the *SMG1* and *ABL* gene (reference gene) were extracted from the previous study (17). Accuracy and specificity of primers were checked using NCBI BLAST tool (HTTP://www.ncbi.nlm.nih.gov/tools/primer-blast/). The applied primers are shown in Table 1.

SMG1 expression levels were measured using the quantitative reverse-transcription PCR (qRT-PCR) with the SYBR® Premix Ex Taq™ II (Takara Bio Inc, Japan) kit in an ABI thermal cycler system. First, 10 µl of master mix (2x), 0.7 µl of each forward and reverse primers, 7 µl of ddH_2O and 2 µl of the cDNA were mixed. The reaction mixture was started with a primary denaturation at 95˚C for 30 seconds, then followed by 40 cycles including 95˚C for 5 seconds, 60˚C for 30 seconds and 72˚C for 30 seconds. Melting program was performed under the following condition: 95˚C for 15 seconds, 60˚C for 1 minute and 95˚C for 15 seconds. Relative expression level of *SMG1* mRNA in the test sample was calculated and normalized to the reference *ABL* mRNA transcript level, as an internal control gene. The equation: relative mRNA expression=$2^{-\Delta\Delta Ct}$ was used for calculation of mRNA expression in each sample (21).

Statistical analysis

Statistical analysis was calculated using SPSS 21.0 (IBM, USA) and JMP10.0 (SAS Institute Inc, USA). Chi-Square and contingency table test were carried out to compare the difference in methylation status between study groups. Kruskal Wallis and means comparisons test using Dunn method by Bonferroni correction, were carried out to compare the expression difference between each phase of CML and control group. Association of *SMG1* methylation status and the corresponding gene expression level was also done by Kruskal Wallis test via investigation of expression difference in various methylation statuses. Association between *SMG1* promoter methylation and hematologic parameters was analyzed using the Kruskal Wallis test with Post hoc tests. Pearson correlation was applied to assess the relationship between mRNA expression level and hematologic parameters. The graphs were drawn using JMP10.0 and GraphPad Prism 9.00 (GraphPad Software Inc, USA). For all analyses, the P values were two-tailed and a $P<0.05$ was considered statistically significant.

Results

SMG1 methylation status

Methylation status of the *SMG1* gene promotor was determined using bisulfite treatment and subsequent MS-PCR. The representative products of *SMG1* methylation for the U and M alleles are illustrated in Table 1. The subjects with a U allele alone are described as unmethylated, the subjects with an M allele alone are described as methylated, and the subject with both M and U alleles are described as the hemimethylated promoters.

All healthy subjects and CMR group participants were unmethylated in the *SMG1* gene promoter. The *SMG1* promoter was methylated in 60% of new case CML patients, while 30% and 10% of patients were hemimethylated and unmethylated in the *SMG1* gene promoter, respectively. In the BP group, 50% of patients were methylated and 50% were hemimethylated in the *SMG1* gene promoter. Therefore, BP and the new case group had hypermethylated gene promoters compared to the control group (P=<0.0001, Fig.1).

Table 1: Sequences of the primers used in this study

Techniques	Primer name	Primer sequences (5´-3´)	Product size (bp)
MS-PCR	*SMG1 M*	F: GCGTACGTGAATTTAAGGGTAC	178
		R: AACAAAAAATCTCCACTACTACGAC	
	SMG1 U	F: GGTGTATGTGAATTTAAGGGTATGT	178
		R: AACAAAAAATCTCCACTACTACAAC	
QR-PCR	SMG1 RNA	F: GGTGGCTCGATGTTACCCTC	106
		R: CTGCGTGAGCGAAGGTTTC	
	ABL RNA	F: TGGAGATAACACTCTAAGCATAACTAAAGG	124
		R: GATGTAGTTGCTTGGGACCCA	

M; Methylated primer, and U; Unmethylated primer, MS-PCR; Methylation-specific polymerase chain reaction, and QR-PCR; Quantitative reverse-transcription polymerase chain reaction.

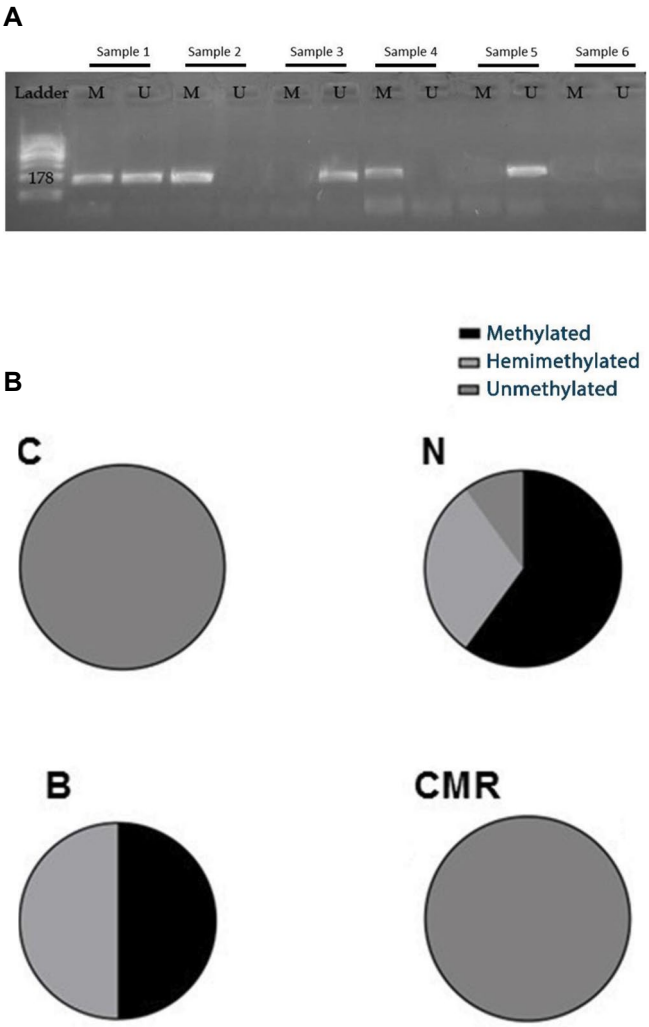

Fig.1: Methylation status of *SMG1* in CML. **A.** Representative gel electrophoresis of PCR products for unmethylated (U) and methylated (M) alleles of *SMG1* in the hemimethylated sample (sample 1), M sample (sample 2), U sample (sample 3), negative control (sample 6), as well as unmethylated primer control (sample 5), and methylated primer control (sample 4). **B.** The pie chart represents proportion (%) of the *SMG1* methylation status in different groups of the current study. CML; Chronic myeloid leukemia, PCR; Polymerase chain reaction, BP; Blastic phase, CMR; Complete molecular response, N; New case, and C; Control.

Baseline and demographic features of patients and healthy subjects are represented in Table 2. There was no significant association between the methylation status of the *SMG1* gene promoter and the hematologic parameters, including hemoglobin level, platelet count and WBC count (Table 3).

Expression level of *SMG1*

qRT-PCR was performed to determine expression level of *SMG1*. Expression level of *SMG1* in the new case group was decreased compared to the control group (P=0.0295). Expression level of *SMG1* in the CMR group was significantly higher than the control group (P=0.04). On the other hand, expression level in the BP group was decreased compared to the control group (P=0.0028). Expression level of *SMG1* in BP group was lower than the new case group (P=0.047). The highest expression level was seen in the CMR group, while the lowest expression level was observed in the BP group (Fig.2).

Table 2: Baseline and demographic features of patients and healthy subjects

Property	CML patients (n=30)	Healthy control (n=10)
Gender	Male=12 (40%)	Male=5 (50%)
	Female=18 (60%)	Female=5 (50%)
Age (Y)	30.5 ± 14.1	28 ± 5.1
WBC count ($\times 10^9$/L)	60 ± 10.2	7.5 ± 1.4
Platelet count ($\times 10^9$/L)	3440 ± 227.499	312 ± 49
Hemoglobin (g/dL)	11.26 ± 2.3	13.6 ± 2.8

CML; Chronic myeloid leukemia and WBC; White blood cell. Data are presented as mean ± SD.

Table 3: Association of methylation as well as correlation of *SMG1* gene expression with hematologic parameters. No significant data was seen

Index	P value	
	Methylation status	mRNA expression (co-efficiency)
WBC count	0.6250	0.2771 (-0.289)
Platelet count	0.5738	0.9424 (0.02)
Hemoglobin	0.2898	0.5883 (0.147)

WBC; White blood cell.

Association of *SMG1* expression and methylation status

Statistical analysis showed that mRNA expression of *SMG1* gene promotor from CML patients negatively associated with methylation status of promoter of this gene (P<0.0001). Methylated samples from new case and blastic groups showed the lowest expression levels, while the unmethylated samples from different study groups had the highest expression level. Hemimethylated samples from the BP or new case groups represented an intermediate expression level, compared to methylated and unmethylated samples.

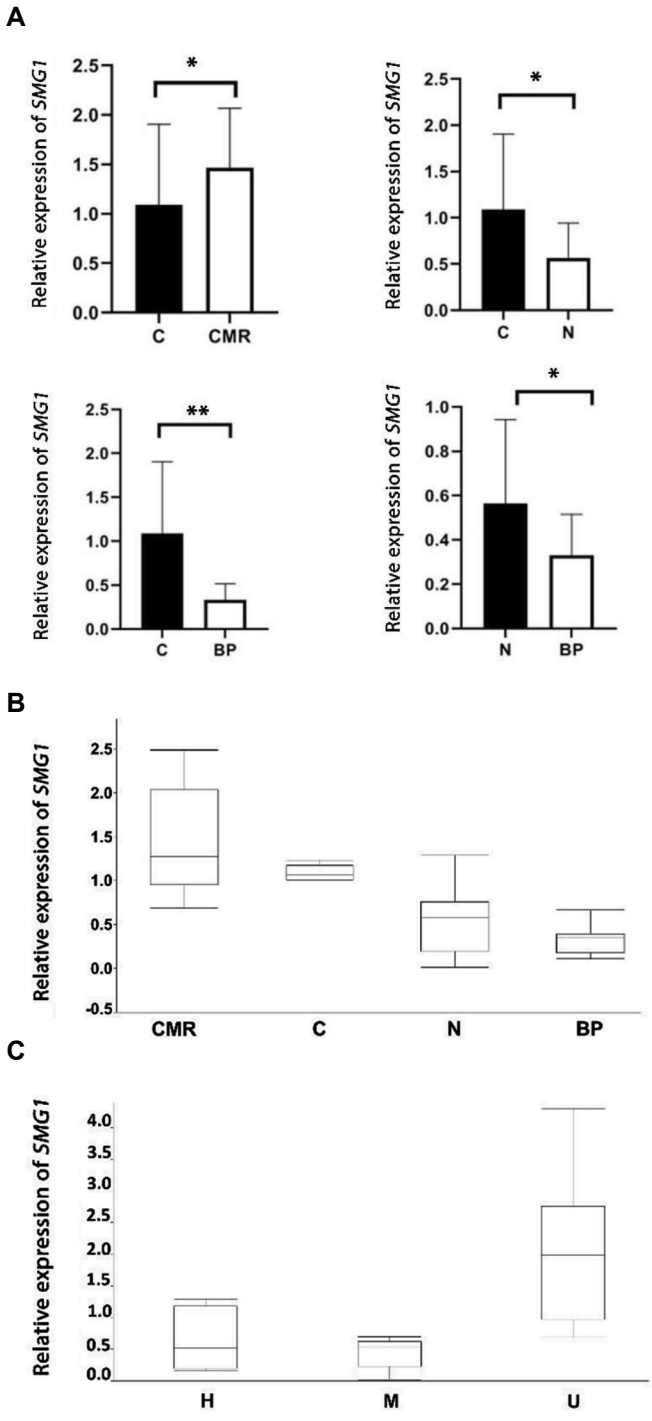

Fig.2: Relative mRNA expression level of the *SMG1* gene CML. **A.** Adual comparison of different groups, **B.** The box plot representations of different groups, and **C.** The box plot representations of different status. CML; Chronic myeloid leukemia, BP; Blastic phase, CMR; Complete molecular response, N; New case, C; Control; H; Hemimethylated, M; Methylated, and U; Unmethylated, *; P<0.05, and **; P<0.01.

Discussion

Epigenetic modifications have recently drawn important attention in the field of cancer etiopathology (22). In the current study, we were interested in the impact of possible aberrant methylated *SMG1* promotors on CML pathogenesis. A bulk of studies have focused on the role of aberrant DNA methylation in CML propagation and subsequent acquired resistance to therapy (10, 23).

SMG1 is a well-known member of the phosphatidylinositol-3 kinase-related kinases (PIKK) family which encompasses important regulating factors, such as mammalian target of rapamycin *(mTOR*) and ataxia telangiectasia mutated (*ATM*) (24). While contribution of *SMG1* in NMD process are well-established, a novel tumor suppressor activity of *SMG1* has been brought to the spotlight (25). *SMG1* activities are essential for genome maintenance and telomere integrity in response to radiation, hypoxia and stress. Preservation from tumor necrosis factor-α (TNF-α)-dependent apoptosis, lifespan adjustment, p53 activation and regulation of diverse genes are some critical anti-tumor activities of *SMG1* in normal cells. Furthermore, DNA damage response (DDR) process is highly dependent on SMG1 activity to protect cells from genetic instability (15, 26-29). In this regard, major research has recently been focused on *SMG1* dysregulation in diverse cancer development. Gubanova et al. (30) revealed a significant reduction in p53 activity in response to radiation in *SMG1*-absent osteosarcoma cell line compared to *SMG1*-wildtype cells. Researchers also demonstrated a deformed response following exposure to genotoxic stress in the cells with loss of *SMG1* activity. According to the multiple studies, complete loss of *SMG1* activity is embryonically lethal; however, deficient mice or *SMG1* haploinsufficiency potently develop inflammation and cancers, especially hematologic malignancy compared to the normal cells. Wong et al. (31) represented *SMG1* mutant variants that lead to the dysfunctional activity of translational proteins as potent pancreatic cancer susceptibility genes. *SMG1* downregulation was reported in hepatocellular carcinoma patients, especially in the end stage disease. Additionally, researchers suggested *SMG1* downregulation as a favorable biomarker for the hepatocellular carcinoma prognosis (32). Hypermethylation and subsequent downregulation of *SMG1* gene promotor have been shown in head and neck squamous cell carcinoma and acute myeloblastic leukemia (AML) (16, 17). It was recently revealed that *SMG1* expression was decreased in chronic lymphoblastic leukemia (CLL) patients, which might be exerted through aberrant promoter hypermethylation (18). Our results, in accordance with these findings, revealed a significant hypermethylation of *SMG1* promotor and subsequent downregulation in CML patients compared to the healthy controls and CMR-CML patients.

Based on the findings of this study, hypermethylation of *SMG1* might play a prognostic role in CML pathogenesis and blastic transformation. Although all CML patients carry similar translocation, they have remarkable clinical heterogeneity. Accumulation of aberrant DNA methylation might associate with clinical heterogeneity and further CP transformation to the BP-CML (8). In our study, both the control and CMR groups showed an unmethylated pattern; however, the expression level of *SMG1* in the CMR group was higher than the control group. While the *SMG1* promoter in the new case group

and BP group were hypermethylated, the BP group had lowest expression level in our study.

Regarding the crucial tumor suppressor activity of *SMG1*, these data suggested that as well as the methylation, other mechanisms participate in *SMG1* expression modulation. In this regard, it has recently been demonstrated that an upregulated microRNA (miR)-18a in nasopharyngeal carcinoma correlated with larger tumor size and further disease propagation. The oncogenic impact of miR-18a is exerted by *SMG1* suppression, which is highly downregulated in the final stages of cancer (33). Furthermore, a devastating role of miR-32 upregulation in ovarian cancer was shown, whose oncogenic role was also applied by suppressing *SMG1* expression (34). In pancreatic cancer, tumor suppressor activity of *SMG1* was suppressed by miR-192 and miR-215, whose downregulation led to the tumor proliferation decline (35). Ectopic expression of miR-585, which was reported to be downregulated in non-small cell lung cancer carcinoma, exerts a tumor suppressor activity by targeting *SMG1* (36). Besides the microRNAs, the potential impact of long noncoding RNAs (lncRNAs) on *SMG1* regulation has recently been shown. lncRNA MAGI2-AS3 exerted tumor suppresser activity in HCC, and its overexpression could limit tumor growth through several mechanisms, including *SMG1* higher expression (37).

Altogether, a significant decline in *SMG1* activity is essential for cancer development that aligns with the reversible epigenetic modifications and provides a brilliant opportunity for cancer treatment (16). It has been demonstrated that CML treating drugs like Imatinib had an indirect effect on methylation status that might recruit candidate genes for anti-tumor activity, such as *SMG1* for boosting treatment response (23). Zhang et al. (38) demonstrated that hepatic cancer cell line treatment with AZD5369, a post-translational modifier agent, induced *SMG1* activation and cancer growth suppression. These findings suggested that *SMG1* was one of the breakthrough targets in cancer therapy. Furthermore, AML cells treatment with Decitabine, a demethylating agent, showed treatment response, including tumor growth inhibition and apoptosis of leukemic cells. Knockdown of the *SMG1* gene destroyed the therapeutic activity of Decitabine, indicating the *SMG1*-dependence role of Decitabine in cancer treatment (17). As a histone modifier agent, Curcumin was is also treated with ovarian cancer and showed therapeutic advantage due to *SMG1* mediating pathways (39). As a result, it is not beyond the expectation that reviving *SMG1* anti-tumor activity with demethylating agents or other regulating ways in CML patients potentially exert treatment advantages. Further research is needed to reach a comprehensive overview of diverse *SMG1* targeting pathways and their possible effect on cancer therapy.

Conclusion

To the best of our knowledge, our results demonstrated for the first time that hypermethylation of the *SMG1* gene is a common circumstance in CML. Furthermore, there is a significant association between methylation status of *SMG1* and its expression. The current study provided an insight into the understanding of *SMG1* hypermethylation status in CML progress, as a possible blastic transformation prognosis or anti-tumor treatment. Moreover, regarding the outstanding anti-tumor activity of *SMG1*, other *SMG1* regulatory pathways have the potential to be modulated for cancer therapy. However, further research is needed to determine the role of *SMG1*, especially *SMG1* hypermethylation, in the development, prognosis and treatment of CML.

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Authors' Contributions

T.H.; Participated in data collecting and performing all experiments. M.S.; Participated for data collecting and analyzing. A.M.; Participated to qRT-PCR assay performance and data analysis. Sh.R., M.A.; Supervised all steps of study and coordinated experiment. All authors read and approved the final manuscript.

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