SLIT2 promoter hypermethylation predicts disease progression in chronic myeloid leukemia

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Abstract

Background: Aberrant DNA methylation plays a crucial role in the progression of myeloid neoplasms. Previously, our literature reported that slit guidance ligand 2 (SLIT2) promoter methylation was associated with disease progression and indicated a poor prognosis in patients with myelodysplastic syndrome. Herein, we further investigated the clinical implications and role of SLIT2 promoter methylation in patients with chronic myeloid leukemia (CML).

Methods: The level of SLIT2 promoter methylation was determined in 104 CML patients, and its clinical significance was analyzed. Moreover, demethylation studies were performed in K562 cells to determine the epigenetic mechanism by which SLIT2 promoter methylation is regulated in CML.

Results: The level of SLIT2 promoter methylation was similar between CML patients and controls. However, deeper analysis revealed that the SLIT2 promoter methylation level in the accelerated phase (AP) and blast crisis (BC) was markedly higher than that in the chronic phase (CP) and controls. Additionally, a marked difference was identified between the SLIT2 promoter hypermethylated and non-hypermethylated groups among CML patients grouped by clinical stage. The frequency of SLIT2 hypermethylation was markedly increased with the progression of clinical stage, that is, it was the lowest in CP samples (12/80, 15%), higher in AP samples (4/8, 50%) and the highest in BC samples (11/16, 69%). Importantly, the level/density of SLIT2 promoter methylation was significantly higher in the advanced stage than in the early stage among the 6 tested paired CML patients. Epigenetically, the expression of the SLIT2-embedded non-coding genes SLIT2-IT1 and miR-218 expression was decreased in patients with CML. SLIT2 promoter hypermethylated cases had a markedly lower SLIT2-IT1 expression level than SLIT2 promoter non-hypermethylated cases. Moreover, SLIT2-IT1 and miR-218 expression was remarkably upregulated in a dose-dependent manner after demethylation treatment of K562 cells.
Conclusions: Hypermethylation of the SLIT2 promoter is correlated with disease progression in CML. Furthermore, SLIT2 promoter methylation may function by regulating the expression of the SLIT2-embedded non-coding genes SLIT2-IT1 and miR-218 during CML progression.

Keywords: SLIT2, Methylation, Expression, Progression, Chronic myeloid leukemia

Background
Chronic myeloid leukemia (CML) is initiated by the reciprocal translocation t(9;22)/Philadelphia (Ph) chromosome, which leads to the formation of the BCR:ABL fusion protein with aberrant tyrosine kinase activity [1]. The treatment of CML is also based on the inhibition of aberrant tyrosine kinase activity as targeted therapy [1]. The typical clinical course of CML includes the initial stage of the chronic phase (CP) and the advanced/aggressive stage of the accelerated phase (AP) and blast crisis (BC) during disease progression [1]. Although CML is cytogenetically/genetically homogenous at the earlier stage, considerable genetic and/or epigenetic heterogeneity is identified in the later stage of CML [1, 2]. Cytogenetic and genetic abnormalities are pathogenetically associated with the progression of CML [2, 3]. Recently, aberrant DNA methylation, which plays a crucial role in the progression of CML, has attracted our attention [4, 5].

The slit guidance ligand (SLIT) family members (SLIT1/SLIT2/SLIT3) are highly conserved secreted glycoproteins that regulate various physiologic processes, such as neuronal axon guidance, cell proliferation, cell migration, and vascularization, by binding to roundabout (ROBO) receptors (ROBO1/ROBO2/ROBO3/ROBO4) [6]. The SLIT/ROBO signaling pathway was originally recognized in the nervous system and functions in neuronal axon guidance and is also considered an important regulator of multiple physiological and oncogenic processes [6, 7]. Recently, an increasing number of studies have reported the dysregulation of SLIT/ROBO signaling pathways in a variety of human cancers [7]. Epigenetic silencing of SLITs mediated by promoter hypermethylation plays a vital role in cancer initiation and progression [10]. Accordingly, a number of studies have shown that SLITs/ROBOs are frequently downregulated and have anticancer roles in the advanced stage of several solid tumors [8, 9]. However, several other studies have demonstrated an oncogenic role during cancer development [8, 9]. Interestingly, the SLIT2-embedded non-coding RNA (ncRNA) miR-218 was found to be downregulated and to act as a tumor suppressor gene in human cancers in most studies [11]. In addition, the other SLIT2-embedded ncRNA SLIT2-IT1 has rarely been investigated.

Previously, our study reported that hypermethylation of the SLIT2 promoter was associated with disease progression in myelodysplastic syndrome (MDS) and predicted poor clinical outcome in both MDS and acute myeloid leukemia (AML) [12]. Moreover, SLIT2 promoter methylation exerted its function by repressing the expression of two SLIT2-embedded ncRNAs, SLIT2-IT1 and miR-218 (SLIT2-IT1/miR-218), in MDS and AML [12]. However, the pattern and clinical implications of SLIT2 promoter methylation in CML remain poorly defined. Herein, on the basis of previous research, we further determined the pattern, clinical implication and role of SLIT2 promoter methylation in patients with CML.

Materials and methods
Subjects and samples
The current study included 104 de novo CML patients (80 in CP stage, 8 in AP stage and 16 in BC stage) and 51 healthy donors (age and sex-matched). The diagnosis and clinical stages of CML were established by clinical manifestation and laboratory examination of peripheral blood (PB)/bone marrow (BM), and were confirmed by molecular detection of the BCR:ABL1 transcript. The BCR:ABL1 transcript detection was quantified using real-time quantitative PCR (RT-qPCR) established previously [13]. BM samples collected from the subjects were further used for the extraction of BM mononuclear cells (BMMNCs) using Lymphocyte Separation Medium (Solarbio, Beijing, China) by gradient centrifugation.

Cell line, cell culture and demethylation treatment
The human CML cell line K562 was cultured in RPMI 1640 medium (Solarbio, Beijing, China) with 10% fetal calf serum (ExCell, Shanghai, China) and grown in a 5% CO2 humidified atmosphere at 37 °C. For demethylation treatment, K562 cells were treated with 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma–Aldrich, St. Louis, MO) at final concentrations of 0 μM, 1 μM, 2 μM, and 4 μM for 3 days. All treated cells were cultured until harvested for extraction of total RNA and DNA.

RNA isolation, reverse transcription and RT-qPCR
Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by reverse transcription to synthesize cDNA for miRNA and long non-coding RNA (lncRNA) detection [12, 14]. RT-qPCR was performed to examine SLIT2-IT1/miR-218 expression by AceQ qPCR SYBR Green Master Mix (Vazyme Biotech
Co., Piscataway, NJ). The primers for SLIT2-IT1/miR-218 expression were previously reported [12]. Relative SLIT2-IT1/miR-218 transcript expression was calculated using the 2−∆∆CT formula according to the ABL1 transcript.

**DNA isolation, chemical modification and RT-qMSP**

The isolation and modification of genomic DNA was performed using Puregene Blood Core Kit B and Epi-Tect Bisulfite Kit (QIAGEN, Duesseldorf, Germany) as described previously [15, 16]. Real-time quantitative methylation-specific PCR (RT-qMSP) was first used to evaluate SLIT2 promoter methylation with AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ). The primers for SLIT2 promoter methylation detection were as reported [12]. Relative SLIT2 promoter methylation was counted using the 2−∆∆CT formula as referred to ALU methylation.

**BSP**

Bisulfite sequencing PCR (BSP) was further performed to detect SLIT2 promoter methylation using TaKaRa Taq™ Hot Start Version (Tokyo, Japan). The primers for SLIT2 promoter methylation detected by BSP were reported previously [17]. The details of BSP can be found in our previous study [17]. Six independent clones from each specimen were selected for Sanger sequencing (BGI, Shanghai, China).

**Statistics**

Statistics were accomplished using SPSS 20.0 and GraphPad Prism 5.0 software packages. The differences in continuous variables between the two groups were compared by Mann–Whitney’s U test. The differences in categorical variables between the two groups were compared by Pearson Chi-square analysis or Fisher’s exact test. The association of SLIT2 promoter methylation with SLIT2-IT1/miR-218 expression was analyzed by Spearman correlation test. Among all statistical analyses, a two-tailed P value < 0.05 was considered statistically significant.

**Results**

**SLIT2 promoter methylation in CML patients**

Previously, we reported the pattern of SLIT2 promoter methylation in patients with MDS and AML and revealed that SLIT2 promoter methylation was correlated with disease progression [12]. Herein, we further detected SLIT2 promoter methylation in CML patients by RT-qMSP as previously described. The results showed that the SLIT2 promoter methylation level was similar between CML patients and controls (P = 0.187, Fig. 1). However, further analysis revealed that the SLIT2 promoter methylation level in the CML-AP and CML-BC stages was markedly higher than that in the CML-CP stage (P = 0.014 and < 0.001, respectively, Fig. 1) and in controls (P = 0.022 and < 0.001, respectively, Fig. 1). The above results indicated that SLIT2 promoter methylation is correlated with an advanced stage of CML and may correlate with disease progression.

**Association between SLIT2 promoter methylation and clinicopathological characteristics of CML patients**

To determine the correlation between SLIT2 promoter methylation and clinicopathological characteristics of CML, the whole cohort of CML patients was divided into two groups based on the previously set cut-off points [12]. No statistical differences were found between the SLIT2 promoter hypermethylated and non-hypemethylated groups with respect to sex, age, hemoglobin, karyotype and BCR-ABL transcript status (Table 1). However, SLIT2 promoter hypermethylated cases exhibited lower white blood cells (WBCs) and platelets than SLIT2 promoter non-hypemethylated cases (P < 0.001 and < 0.006, respectively, Table 1). Notably, a marked difference was identified between the SLIT2 promoter hypermethylated and non-hypemethylated groups in CML patients grouped by clinical stage (P < 0.001, Table 1). The frequency of SLIT2 hypermethylation was markedly increased with the progression of clinical stage, that
is, it was the lowest in CML-CP samples (12/80, 15%), higher in CML-AP samples (4/8, 50%) and the highest in CML-BC samples (11/16, 69%) \( (P < 0.001, \text{Table 1}) \). These results further confirmed that \( SLIT2 \) promoter methylation was correlated with an advanced stage of CML and may correlate with disease progression.

**SLIT2 promoter methylation alteration during disease progression in paired CML patients**

Given the results above, we hypothesized that \( SLIT2 \) promoter methylation was correlated with disease progression in CML. To test this hypothesis, we further examined \( SLIT2 \) promoter methylation in paired CML patients during disease progression. By RT-qMSP, the level of \( SLIT2 \) promoter methylation was significantly upregulated in the advanced stage compared with the early stage among the tested 6 paired CML patients (Fig. 2). Moreover, the \( SLIT2 \) promoter methylation density in these paired patients was further detected by BSP (Fig. 3) and was closely correlated with the results detected by RT-qMSP \( (R=0.895, P<0.001, \text{Additional file 1: Fig S1}) \). Taken together, these results suggest that \( SLIT2 \) promoter methylation is correlated with disease progression in CML.

**Epigenetic regulatory effects of \( SLIT2 \) promoter methylation in CML**

Previously, we revealed that \( SLIT2 \) promoter methylation was associated with \( SLIT2 \)-embedded ncRNAs \( SLIT2-IT1/miR-218 \) expression but not \( SLIT2 \) expression in MDS and AML. Herein, we further detected \( SLIT2-IT1/miR-218 \) expression in 51 CML patients with available mRNA samples matched to DNA samples. \( SLIT2-IT1 \) expression was markedly decreased \( (P=0.030, \text{Fig. 4a}) \), whereas \( miR-218 \) expression was nearly undetectable in CML patients. Moreover, although \( SLIT2-IT1 \) expression exhibited a weak negative association with \( SLIT2 \) promoter methylation in CML patients \( (R=-0.289, P=0.039, n=51) \), cases with \( SLIT2 \) promoter hypermethylation had a markedly lower \( SLIT2-IT1 \) expression level than those without \( SLIT2 \) promoter hypermethylation \( (P=0.004, \text{Fig. 4b}) \). To further

| Patients' parameters | \( SLIT2 \) promoter non-hypermethylated \( (n=77) \) | \( SLIT2 \) promoter hypermethylated \( (n=27) \) | \( P \)-value |
|----------------------|-----------------------------------------------|-----------------------------------------------|------------|
| Sex, male/female     | 50/27                                         | 15/12                                         | 0.489      |
| Median age, years (range) | 51 (15–88)                                   | 46 (20–75)                                   | 0.680      |
| Median WBC, \( \times10^9/L \) (range) | 128.4 (31.5–413.8)                           | 41.6 (21.7–293.4)                             | <0.001     |
| Median hemoglobin, g/L (range) | 101 (57–146)                                 | 92 (50–152)                                  | 0.277      |
| Median platelet, \( \times10^9/L \) (range) | 387 (22–1489)                                | 250 (16–914)                                 | 0.006      |
| Karyotype            |                                               |                                               | 0.438      |
| t(9;22)              | 53 (69%)                                      | 14 (52%)                                      |            |
| t(9;22) with additional alteration | 10 (13%)                                      | 4 (15%)                                      |            |
| Normal karyotype     | 4 (5%)                                        | 3 (11%)                                      |            |
| No data              | 10 (13%)                                      | 6 (22%)                                      |            |
| Clinical stage       |                                               |                                               | <0.001     |
| CP                   | 68 (88%)                                      | 12 (44%)                                     |            |
| AP                   | 4 (5%)                                        | 4 (15%)                                      |            |
| BC                   | 5 (7%)                                        | 11 (41%)                                     |            |
| \( BCR::ABL1 \) transcript (relative copy) | 210 (16.9–3784.8)                            | 239.1 (13.8–14464.7)                         | 0.366      |

\( WBC \) white blood cell, \( CP \) chronic phase, \( AP \) accelerated phase, \( BC \) blast crisis

**Table 1** Comparison of clinicopathological characteristics between \( SLIT2 \) hypermethylated and non-hypermethylated CML patients
verify the epigenetic regulatory effects of SLIT2 promoter methylation on the ncRNAs SLIT2-IT1/miR-218, we performed demethylation treatment of the CML cell line K562 with 5-aza-dC. With the decreased density of SLIT2 promoter methylation, SLIT2-IT1/miR-218 expression was markedly upregulated in a dose-dependent manner after 5-aza-dC treatment (Fig. 4c–f). Collectively, these results support the epigenetic regulatory effects of SLIT2 promoter methylation on the expression of SLIT2-embedded ncRNAs SLIT2-IT1/miR-218 in CML.

Discussion
The SLIT/ROBO signaling pathway has been implicated in the regulation of developmental processes and physiological processes [6, 7]. SLIT/ROBO signaling plays crucial roles in a number of cell signaling pathways including axon guidance, angiogenesis, cell proliferation, cell apoptosis and cell motility [6, 7]. Moreover, inactivation of SLITs/ROBOs expression mediated by promoter methylation in cells can lead to cancer initiation and progression [10]. Notably, several studies have demonstrated that SLITs/ROBOs are frequently downregulated in the advanced stage of various solid tumors [8, 9]. This evidence indicated that the SLIT/ROBO signaling pathway may play a crucial role in cancer progression than cancer initiation. Previously, we reported that SLIT2 promoter methylation through the inactivation of SLIT2-IT1/miR-218 expression may play a key role in MDS progression by affecting cell proliferation, apoptosis and colony formation both in vitro and in vivo [12]. In the current study, we observed that SLIT2 promoter hypermethylation was associated with lower WBCs and platelet in CML, which suggests that SLIT2 promoter hypermethylation may be associated with hematopoietic stem cells differentiation fate. Accordingly, further functional studies are needed to determine the direct role of aberrant SLIT2 promoter methylation in leukemogenesis during CML progression.

To date, the mechanisms involved in CML progression have been preliminarily identified. Cytogenetic aberrations, such as double t(9;22)/Ph chromosome, trisomy chromosome 8, i(17q), trisomy chromosome 19, t(3;21) and t(7;11), and molecular alterations, including TP53 mutations, RAS mutations and increased BCR::ABL1 transcript levels, are pathogenetically correlated with the progression of CML [2, 3]. Moreover, epigenetic alterations, such as aberrant DNA methylation, have also been identified to play a vital role in the disease evolution of CML [4, 5]. For instance, Li et al. revealed that SHP-1 hypermethylation was involved in CML evolution.
Fig. 4 Transcriptional regulatory effects of SLIT2 promoter methylation on SLIT2-IT1/miR-218 expression in CML. 

(a) Relative SLIT2-IT1 expression level in CML patients.
(b) Relative SLIT2-IT1 expression between SLIT2 promoter non-hypermethylated and hypermethylated groups.
(c) SLIT2-IT1 expression before and after 5-aza-dC treatment with different dose.
(d) miR-218 expression before and after 5-aza-dC treatment with different dose.
(e) SLIT2 promoter methylation density before 5-aza-dC treatment.
(f) SLIT2 promoter methylation density after 5-aza-dC treatment (4 μM).
through the regulation of the BCR::ABL1, AKT, MAPK, MYC and JAK2/STAT5 signaling pathways [18]. Additionally, our research group has also revealed the correlation of SOX30, ID4 and DLX4 hypermethylation with disease progression in CML [19–21]. A recent study demonstrated that promoter hydroxymethylation of tumor suppressor genes DAPK1, RIZ1, P16INK4A, RASSF1A and p14ARF was a characteristic feature of CML disease progression and indicated poor imatinib response and poor overall survival of CML patients to imatinib therapy [22]. On the basis of our previous study [12], we further investigated SLIT2 promoter methylation in another myeloid malignancy CML. In accordance with the results in MDS [12], SLIT2 promoter methylation was also correlated with advanced clinical stage of CML, and played a crucial role in disease progression. Interestingly, Heller et al. observed up to 897 genes that were methylated at the time of progression but not at the time of diagnosis in CP-CML patients who progressed to AP/BC-CML using next-generation sequencing [4]. However, SLIT2 promoter hypermethylation was not identified in this study [4], which may be attributed to differences in ethical considerations. Since this is the first report of SLIT2 promoter hypermethylation in CML progression, prospective investigations are needed to confirm and expand our results.

DNA hypermethylation mainly functions by inactivating gene expression in cancer development. Although a few investigations have demonstrated the association between SLIT2 promoter methylation and SLIT2 expression in some types of solid tumors [23], our recent study revealed that SLIT2 promoter methylation was correlated with the expression of SLIT2-embedded ncRNAs SLIT2-IT1/miR-218 but not with SLIT2 in AML [12]. Herein, we also explored the expression of SLIT2-IT1 and miR-218-218 expression in CML. The results showed that SLIT2-IT1/miR-218 was significantly decreased in CML patients, and was negatively correlated with SLIT2 promoter methylation. Moreover, demethylation studies also confirmed the epigenetic mechanism of SLIT2 promoter methylation in regulating ncRNAs SLIT2-IT1/miR-218 expression in CML. Taken together, these results indicated that SLIT2 promoter hypermethylation may function by repressing SLIT2-IT1/miR-218 expression during CML progression.

Conclusion

Hypermethylation of the SLIT2 promoter is correlated with disease progression in CML. Furthermore, SLIT2 promoter methylation may regulate the expression of SLIT2-embedded non-coding genes SLIT2-IT1/miR-218 during CML progression.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

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Additional file 1: Figure S1. Correlation between SLIT2 methylation density detected by BSP and SLIT2 methylation level detected by RT-qMSP.
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