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# Long noncoding RNA MEG3 inhibits proliferation of chronic myeloid leukemia cells by sponging microRNA21

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ABSTRACT

The long noncoding RNA (lnc) maternally expressed 3 (MEG3) is downregulated in many types of cancers. However, the relationship between lncRNA MEG3, microRNA-21 (miR-21) and chronic myeloid leukemia (CML) blast crisis is unknown. This study examined bone marrow samples from 40 CML patients and 10 healthy donors. Proliferation and apoptosis assays, real-time polymerase chain reaction (PCR), bisulfite sequencing PCR, Western blotting, luciferase assay, RNA pull-down, RNA immunoprecipitation (RIP), co-immunoprecipitation (CoIP) and Chromatin immunoprecipitation (ChIP) were performed. We found that MEG3 and PTEN expression were down-regulated, whereas, MDM2, DNMT1 and miR-21 were up-regulated in the accelerated and blast phases of CML. Treated with 5-azacytidine decreased the level of MDM2, DNMT1 and miR21, but increased the level of MEG3 and PTEN. Overexpression of MEG3 and silencing the expression of miR-21 inhibited proliferation and induced apoptosis. MEG3 overexpression and silencing the expression of miR21 influence the levels of MMP-2, MMP-9, bcl-2 and Bax. MEG3 was able to interact with MDM2 and EZH2. MDM2 could interact with DNMT1 and PTEN. MYC and AKT can interact with EZH2. ChIP-seq showed that the promoter of KLF4 and SFRP2 interacts with DNMT1. In conclusion, lncRNA MEG3 and its target miR21 may serve as novel therapeutic targets for CML blast crisis; and demethylation drugs might also have potential clinical application in treating CML blast crisis.

## 1. Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy that is characterized by the Philadelphia chromosome, which forms the breakpoint cluster region (BCR)/Abelson murine leukemia (ABL) fusion gene, which encodes the P210 BCR/ABL1 protein [1,2]. Key pathways are associated with CML [3]. Typically, CML patients pass through three phases: a chronic phase (CP), an accelerated phase (AP) and a blast phase (BP) [4]. Although tyrosine kinase inhibitors (TKIs) treatment can improve the 5-year survival rate of CML patients, patients who are resistant to TKIs or who receive no drug treatment will eventually progress to the advanced phases (AP and BP) in approximately 3–5 years. Unfortunately, even if these patients receive standard chemotherapy regimens, their prognosis remains poor [5]. Thus, it is important to determine the underlying molecular and biological mechanism of CML blast crisis and important to find novel treatment targets for CML blast crisis.

Long noncoding RNAs (lncRNAs) have been reported to be key regulators of important biological processes that play roles in specific cancers and in other diseases [6]. Noncoding RNAs (ncRNAs) are generally classified into two groups according to their length, namely, small ncRNAs (sncRNAs) and lncRNAs. SncRNAs are less than 200 nt long, and lncRNAs are transcripts that are longer than 200 nucleotides; neither has protein-coding functions [7,8]. LncRNAs are differentially expressed in solid tumors and hematopoietic tumors [9,10]. LncRNA maternally expressed 3 (MEG3) is associated with many cancers, such as colorectal cancer [11], ovarian cancer [12], and hepatocellular carcinoma [13], MEG3 is involved in imatinib resistance in CML and possibly contributes to imatinib resistance by regulating miR-21 [14], however, the relationship between MEG3 and the molecular biological mechanism underlying the blastic transformation of CML is still not clear.

Epigenetics involves heritable changes in gene expression, such as DNA methylation and chromatin conformation, without any corresponding changes in the DNA sequence [14,15]. Abnormal DNA promoters are reported to be involved in tumorigenesis. MEG3 methylation has been reported to be associated with many types of cancer, such as ovarian cancers and meningiomas [16,17]; however, the relationship between MEG3 methylation and the blastic transformation of CML remains largely unknown. Studies of the mode of action of lncRNAs have

*Abbreviations:* CML, chronic myeloid leukemia; BSP, bisulfite sequencing PCR; RT-qPCR, real-time quantitative polymerase chain reaction; WB, Western blotting; FCM, flow cytometry

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**Table 1**
Characteristics of the patients included in the study.

| Item | CML-CP (n = 20) | CML-AP (n = 10) | CML-BP (n = 10) |
|---|---|---|---|
| Age (years), median (range) | 43.4(9–65) | 44.1(13–69) | 43.9(20–69) |
| Male/female, (n/n) | 14/6 | 7/3 | 6/4 |
| WBCs × $10^9$/median (range) | 213.4(30.2–517) | 243.5(47.4–396) | 63.5(27.4–224) |
| Hemoglobin level (g/L) | 91(76–120) | 85(61–105) | 64.4(52–79) |
| Platelet count,$10^9$/median (range) | 525(99–809) | 295(52–725) | 45.5(19–71) |

AP, accelerated phase; BP, blast phase; CML, chronic myeloid leukemia; CP, chronic phase; WBC, white blood cells.

shown that a number of lncRNAs can interact with chromatin to regulate gene expression. MEG3 has been reported to interact with polycomb repressive complex 2(PRC2) in many cancer cells lines [18]. The proteins that bind to MEG3 in CML crisis cell lines are unknown.

Similar to lncRNAs, microRNAs (miRNAs) do not encode proteins, but are less than 200 nt in length. MicroRNAs are small, endogenous and noncoding RNA that contribute to the regulation of essential cellular processes, such as proliferation, apoptosis, development, and differentiation [19]. Recent studies have shown that miRNAs are involved in tumorigenesis and function as tumor suppressor genes or oncogenes [20,21]. MiR-21 involved in the progression of lung cancer and may be a novel therapeutic target for the disease [22]. However, the role of miR-21 in the progression of CML is largely unknown.

## 2. Materials and methods

### 2.1. Specimen collection

Bone marrow samples were collected from 40 CML patients, who were admitted to the Department of Hematology of the Second Hospital of Hebei Medical University between May 2015 and June 2017 (Table 1). Bone marrow samples from 10 healthy donors were selected to serve as controls. Bone marrow mononuclear cells were isolated via lymphocyte separation. This study was approved by the Ethics Committee of the Department of Hematology of the Second Hospital of Hebei Medical University, and each patient signed informed consent. The inclusion criteria were (i) diagnosis of CML via bone marrow morphology, immunology, molecular biology and cytogenetic; (ii) clear pathological staging; and (iii) availability of intact clinical data. The exclusion criteria were (iv) significant organ dysfunction; (v) pregnancy (in females); and (vi) failure to provide informed consent. No chemotherapy was administered before the specimens were collected.

### 2.2. Cell culture

KCL22 and K562 cells were maintained in our laboratory. KCL22 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco, Beijing, China), which contained 10% fetal bovine serum (FBS) (Clark Bio, Claymont, DE, USA), 100 units/ml penicillin and 100 μg/ml streptomycin. K562 cells were cultured in RPMI 1640 medium (Gibco, Beijing, China) supplemented with 10% FBS and the two antibiotics listed above.

### 2.3. Cell treatment

5-Azacytidine was purchased from ApexBio, (Houston, TX, USA). KCL22 and K562 cells were seeded in 6-well plates at a density of $1 \times 10^6$ cells per well. MTT assays were performed to detect the EC50 concentrations of 5-azacytidine. K562 and KCL22 were treated with drugs according to the respective EC50 values. KCL22 cells were treated with 60, 80 and 100 μmol/l 5-azacytidine; K562 cells were treated with 40, 60 and 80 μmol/l 5-azacytidine. The KCL22 and K562 cells were treated with 5-azacytidine for 48 h.

### 2.4. Cell transfection

The full-length cDNA sequence of MEG3 was synthesized by Invitrogen and then cloned into the pLVX-hMEG3-ZsGreen-Puro lentiviral overexpression vector, which was constructed as described previously. MiR21 inhibitors miR21 controls and miR21 mimics were also synthesized by Invitrogen (Carlsbad, CA, USA). KCL22 and K562 cells were seeded onto six-well plates ($5 \times 10^5$/well)and then transfected with the LV-MEG3 or LV-control, KCL22 and K562 cells were seeded into 6-well plates and transfected with miR-21 inhibitors, miR-21 controls or miR-21 mimics using lipo3000 (Life Invitrogen, Carlsbad, CA).

### 2.5. MTT assays

We suspended KCL22 and K562 cells and seeded them into 96-well plates ($1 \times 10^5$ cells/well). We cultured them for 0, 24, 48, 72 and 96 h using IMDM medium and 1640 with 10% FBS at 37 °C. The proliferation of KCL22 and K562 cells was determined by using an MTT assay. Briefly, following cell culture, 10 μl 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA, USA) was added to each well and the 96-well plates were incubated at 37 °C in a humidified 5% $CO_2$ atmosphere for 4 h. Absorbance was then read at 490 nm, in a microplate reader (Thermo Fisher USA).

### 2.6. Apoptosis assays

KCL22 and K562 cells were seeded into 6-well plates after transfection and treated according to the manufacturer's instructions. The cells were stained with 5 μl of AnnexinV-APC AnnexinV-FITC and 5 μl of PI (BD Bioscience Pharmingen USA) and then analyzed with a BD FACSCanto II system (BD USA). The apoptosis were performed using BDFAC-Diva (BD, USA).

### 2.7. Bisulfite sequencing PCR

Genomic DNA was isolated using a DNA extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. CpG island methylation was detected by performing bisulfite sequencing PCR (BSP). The CpG islands of MEG3 were predicted using the software Methprimer. CpG islands were found across the promoter and the first exon region, and primers were designed with this region as the target sequence. The primer (Shanghai Generay Biotech Co. Ltd.) sequences were as follows: MEG3 forward, TTTGAATAATAAGAGAAAG TATGA, and reverse, AACTAACTAAATAAAATTTATATAA. The MEG3 product length was 437 bp, which included 10 CG sites. The reaction process was as follows: pre-denaturation at 95 °C for 4 min; modification for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 40 s at 72 °C for 40 cycles, followed by extension at 72 °C for 5 min. PTG19-T (Generay, lot: GV6021) was used as a carrier, and the procedure was performed according to the manufacturer's instructions. The transformation was performed using XL10-Gold ultracompetent cells, and the cells were transformed, recovered and plated according to the product instructions. Positive colonies were identified by enzyme digestion and then sequenced.

### 2.8. Real-time quantitative RT-PCR

Total RNA was isolated with Trizol (Life Technologies, Invitrogen, USA), and the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher, USA) was used to synthesize cDNA according to the manufacturer's instructions. The reaction was performed at 42 °C for 60 min,