

Original Article

Expression of minichromosome maintenance 8 in chronic myelogenous leukemia

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Abstract: Objectives: Minichromosome maintenance 8 (MCM8) is identified as an initiating helicase involved in DNA elongation and involved in cancer. However, little information is available for the role of MCM8 on chronic myelogenous leukemia (CML). We aimed to explore the expression and effect of MCM8 on CML. Methods: Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) were prepared from six patients with CML and three healthy individuals. The mRNA levels of MCM8 were determined and compared. The expression of MCM8 was silenced by small interfering RNA (siRNA) approach in human CML cell line K562. After transfection with MCM8 siRNA, cell viability and apoptotic rate were analyzed, as well as the protein expression levels of Caspase-3 and B-cell lymphoma (Bcl)-xL. Results: Relative mRNA levels of MCM8 were both significantly higher in PBMC and BMMC from CML patients than those in healthy individuals ($P < 0.05$). The cell viability was significantly reduced while the apoptotic rate was statistically increased by knockdown of MCM8 compared to control group or the scramble siRNA group (both $P < 0.05$). Moreover, the protein expression levels of Caspase-3 were significantly increased ($P < 0.05$), and while the levels of Bcl-xL were statistically reduced ($P < 0.05$) compared to the control group or the scramble siRNA group. Conclusion: MCM8 plays a significant role in CML, and knockdown of MCM8 might be a potentially targeted therapy for CML.

Keywords: Chronic myelogenous leukemia, minichromosome maintenance 8, apoptosis

Introduction

Chronic myelogenous leukemia (CML) is a stem-cell driven malignancy characterized by the presence of the Philadelphia chromosome, which results from transformation of a primitive hematopoietic cell by the breakpoint cluster region (BCR)/Abelson murine leukemia (ABL) [1]. CML can occur at any age with the peak age of onset between 50 and 60 years. The annual incidence has been estimated at 1-2 cases/100,000 [1], and the prevalence of CML is increasing [2]. Although most patients with CML have excellent responses to tyrosine kinase inhibitors (TKI) therapy and are expected to live for many years, the quality of life is much lower than their age- and sex-matched controls [3]. In addition, progression to a more advanced stage of the disease still contributes to the leading cause of death for CML, even though with the TKI treatment [4]. Therefore, it

is imperative to develop a potentially targeted and tailored therapy for CML.

Although minichromosome maintenance (MCM) proteins were identified 30 years ago, the family has been paid great attention to research. The family was first identified in budding yeast *Saccharomyces cerevisiae* [5] and then was found to play essential roles in DNA replication [6]. Previous studies have suggested that MCM 2-7 are involved in DNA replication elongation, checkpoint signaling, chromatin remodeling, transcription and prereplication complex formation [7-10]. Recently, overexpression of MCMs have been implicated in multiple cancers such as esophageal squamous cell carcinoma [11], cervical carcinogenesis [12], gastric adenocarcinoma [13], and breast carcinoma [14], making MCM 2-7 complex suitable biomarkers for malignancies. MCM8 and its physical partner MCM9, newly discovered two additional MCM

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family proteins, have been reported to be implicated in DNA replication [15]. In addition, an autosomal recessive pathogenic variant in MCM8 underlies premature ovarian failure (POF) and increase chromosomal instability [16]. MCM8 shares significant homology with human MCM7 and is identified as an initiating helicase involved in DNA elongation [15]. Besides it has reported that it is overexpressed in several cancers [17]. However, little information is available for the expression and effect of MCM8 on CML.

Therefore, the purpose of this study was aimed to explore the roles of MCM8 in CML. Six patients with CML and three healthy individuals were enrolled in our study, and CD34⁺ cells were isolated from both peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC). We determined and compared the mRNA levels of MCM8 in CML and healthy individuals. After transfection with MCM8 small interfering RNA (siRNA) approach in human CML cell line K562, cell viability and apoptotic rate were analyzed. Moreover, related apoptosis mechanism was detected. Our study might provide insight into new targeted therapy for CML.

Materials and methods

Samples collection and separation of leukemic blast cells

Blood and bone marrow (BM) samples were obtained from six patients with CML (4 males and 2 females, aged 26-62 years, chronic phase) and three healthy individuals (2 males and 1 female, aged 24-56 years) between December 2014 and August 2015. The diagnosis of CML was based on standard clinical and hematologic criteria. The positive results of BM histology and cytogenetic analysis (>80% Philadelphia-chromosome-positive cells in BM before mobilization treatment) were required for the diagnosis of CML in chronic phase. The patients had not received imatinib (IM) treatment. The approval of local Ethics Committee and informed consent were obtained for the use of human samples. PBMC and BMMC were prepared from by Ficoll-Hypaque density gradient centrifugation. CD34⁺ cells were isolated from PBMC and BMMC by magnetic-activated cell sorting (MACS) using human CD34 microbead kit (Miltenyi, Munich, Germany). The cells

were stained with PE-conjugated anti-human CD34 monoclonal antibody (Beckman-Coulter, Villepinte, France) and incubated at 4-8°C for 20 min. Then the cells were washed with buffer, incubated with anti-PE microbeads (Miltenyi Biotech) for another 20 min at 4-8°C, and washed with phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) (Sigma, St. Louis, MO). The purity of CD34⁺ was evaluated by fluorescence-activated cell sorting analysis.

Cell culture and transfection

Human CML cell line K562 was obtained from American Type Culture Collection (Manassas, VA, USA). These cells were in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 1% L-glutamine (Life Technologies, Grand Island, NY), 1% penicillin-streptomycin (Life Technologies), and 1% amphotericin B (Life Technologies) at 37°C with 5% CO₂. Cell transfections were performed by small interfering RNA (siRNA) silencing approach using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. A scramble siRNA was used as a negative control. The sequences were listed as follows: siMCM8-ORF, 5'-AGAAGACGCUG-AGGAUAUA-3'; siMCM8-UTR, 5'-CAUAUCAGAU-GUAG GCAUA-3'.

Real-time polymerase chain reaction (PCR)

Total RNA was respectively extracted from PBMC, BMMC and K562 cells by TRIzol reagent (Invitrogen) according to the manufacturer's recommendation. Gene expression levels were measured by real-time reverse transcription PCR using QuantiTect™ SYBR® Green RT-PCR kit (Qiagen). Complementary DNA (cDNA) was synthesized using the Reverse Transcription System (Applied Biosystems, Foster City, Calif). Quantitative PCR data were identified by an ABI Prism 7000 sequence detection system (Applied Biosystems). PCR conditions consisted of 1 cycle of predenaturation at 95°C for 1 min, reverse transcription at 50°C for 20 min, inactivation at 95°C for 15 min, 50 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 25 s, and a final extension at 72°C for 15 s. The mRNA levels were expressed as Cycle threshold (CT). Transcript levels were normalized by subtracting the corresponding glyceral-

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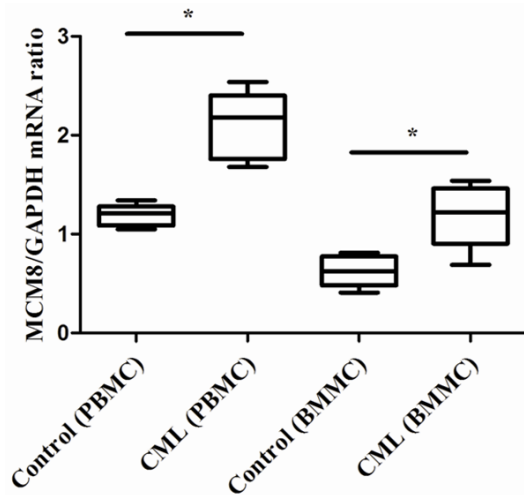


Figure 1. mRNA expression levels of MCM8 in patients with CML. The mRNA expression levels of MCM8 in CD34⁺ cells isolated from PBMC and BMMC are measured by real-time PCR. MCM, Minichromosome maintenance proteins; CML, chronic myelogenous leukemia; PBMC, Peripheral blood mononuclear cell; BMMC, bone marrow mononuclear cell; PCR, polymerase chain reaction. **P* < 0.05 compared to the control group.

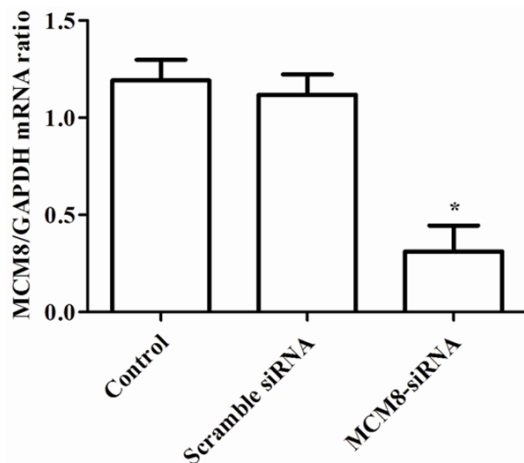


Figure 2. Relative mRNA expression levels of MCM8 after transfection with MCM8 siRNA. The mRNA expression levels of MCM8 in K562 cells transfected with MCM8 siRNA or scramble siRNA are validated by real time PCR. MCM, Minichromosome maintenance proteins; PCR, polymerase chain reaction; siRNA, small interfering RNA. **P* < 0.05 compared to the control group or the scramble siRNA group.

dehydrogenase (GAPDH) gene values. Reactions were run in triplicate.

Cell proliferation assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) according to the manufacturer's protocol. After transfection at 0 h, 72 h, and 7 d, the cells were harvested, seeded in 96-well plates at a final concentration of 1×10^4 per mL, and incubated at 37°C with 5% CO₂. Thereafter, 10 μL MTT was added to each well and incubated at 37°C for another 2 h. Number of viable cells were counted by trypan blue exclusion, followed by detecting absorbance at 550 nm.

Flow cytometry (FCM) assay

For determination of apoptotic cells, an Annexin V-fluorescein-5-isothiocyanate (Annexin V-FITC) apoptosis detection kit (Biovision, USA) was used according to the manufacturer's protocol. Briefly, cells (1×10^6 cells/ml) were harvested after transfection at 0 h, 72 h, and 7 d, seeded in 24 well plates (1.0×10^5 cells), and mixed with 10 μL Annexin. The mixture was incubated in the dark for 15 min at room temperature. After that, 10 μL propidium iodide (PI) was added to each well. After washed in ice cold PBS and immediately analyzed by FCM (Becton Dickinson, San Jose, CA, USA).

Western blot analysis

After transfection at 24 h, cells in each group were collected for protein extraction. The protein density was determined by BCA Protein Assay Kit (Fisher Scientific, PA, USA). Protein samples were resolved with 10-12% sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Milipore, Bedford, MA), blocked with 5% nonfat milk in Tris Buffered Saline with Tween (TBST) for 2 h at room temperature. Thereafter the members were incubated with the following primary antibodies overnight at 4°C: anti-Caspase-3 monoclonal antibody (1:1000, Santa Cruz Biotech) and anti-B-cell lymphoma (Bcl)-xL (1:1000, Santa Cruz Biotech), followed by an appropriate secondary antibody. An anti-human GAPDH antibody (Santa, Cruz Biotechnology) was considered as a loading control. The immunoreactive protein bands were visualized by enhanced chemiluminescence. The results were analyzed using ImageJ software (National Institutes of Health).

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Student's t test (for 2 groups) and one-way analysis of variance (ANOVA) (for ≥ 3 groups) were performed to statistical com-

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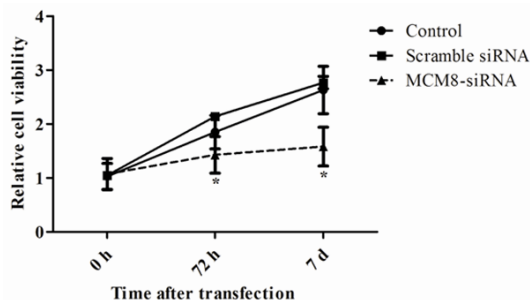


Figure 3. Cell viability after transfection with MCM8 siRNA. We perform MTT assay to detect the cell viability after transfection with MCM8 siRNA (at 0 h, 72 h and 7 d). MCM, Minichromosome maintenance proteins; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA. * $P < 0.05$ compared to the control group or the scramble siRNA group.

parisons. Statistical package for the social sciences (SPSS) (version 19.0; SPSS Inc., Chicago, IL) was used to analyze the results. A statistical significance was defined when $P < 0.05$.

Results

Expression of MCM8 in patients with CML

To investigate the expression of MCM8 in patients with CML, we enrolled six patients with CML and three healthy individuals as a control. Both blood and BM samples were collected from all the participants. CD34⁺ cells were isolated from both blood and bone marrow samples. The mRNA expression levels of MCM8 were measured by real time PCR. As shown in **Figure 1**, the results showed that the relative MCM8 mRNA expression levels either in PBMC and BMMC samples were both significantly higher than those in the healthy individuals (both $P < 0.05$), indicating that MCM8 might play a significant role in CML.

Expression of MCM8 after transfection with siRNA

To examine the role of MCM8 in CML, siRNA technology was used to suppress the expression of MCM8 in K562 cells. After 24 h transfection, the expression levels of MCM8 were validated by real time PCR. The results showed that the relative mRNA expression levels of MCM8 were significantly decreased by transfection with MCM8 siRNA compared to scramble siRNA or control group ($P < 0.05$), suggesting that the expression of MCM8 were successfully silenced. However, no significant differ-

ences were found between cells transfected with scramble siRNA and control group (**Figure 2**).

Cell viability after transfection with MCM8 siRNA

In order to examine the effect of MCM8 on cell viability, MTT assay was performed after transfection with MCM8 siRNA. As shown in **Figure 3**, the cell viabilities at both 72 h and 7 d were significantly reduced by knockdown of MCM8 compared to control group or the scramble siRNA group ($P < 0.05$), indicating that knockdown of MCM8 could effectively suppress cell proliferation of CML.

Apoptosis analyses after transfection with MCM8 siRNA

To further determine the effect of MCM8 on cell apoptosis, FCM assay was carried out after transfection with MCM8 siRNA at 0 h, 72 h and 7 d. The results were showed in **Figure 4A-D**. We found that there were no significant differences in apoptotic cell ratio at 0 h, while the ratio were statistically increased by transfection with MCM8 siRNA at 72 h and 7 d compared to the control group or the scramble siRNA group ($P < 0.05$), demonstrated that knockdown of MCM8 could effectively induce apoptosis in CML.

Expression of Caspase-3 and Bcl-xL after transfection with MCM8 siRNA

We then investigated the apoptotic mechanism induced by silencing of MCM8. The protein expression levels of Caspase-3 and Bcl-xL after transfection with MCM8 siRNA were determined by Western blotting (**Figure 5A-C**). The results showed that after transfection with MCM8 siRNA, the protein expression levels of Caspase-3 were significantly increased ($P < 0.05$), while the protein expression levels of Bcl-xL were statistically reduced ($P < 0.05$) compared to the control group or the scramble siRNA group. The results suggested that silencing of MCM8 promoted apoptosis by regulating the expression of Caspase-3 and Bcl-xL.

Discussion

In the present study, we investigated the role of MCM8 in CML. Our results show that the expression of MCM8 is significantly higher in patients with CML than healthy controls. In

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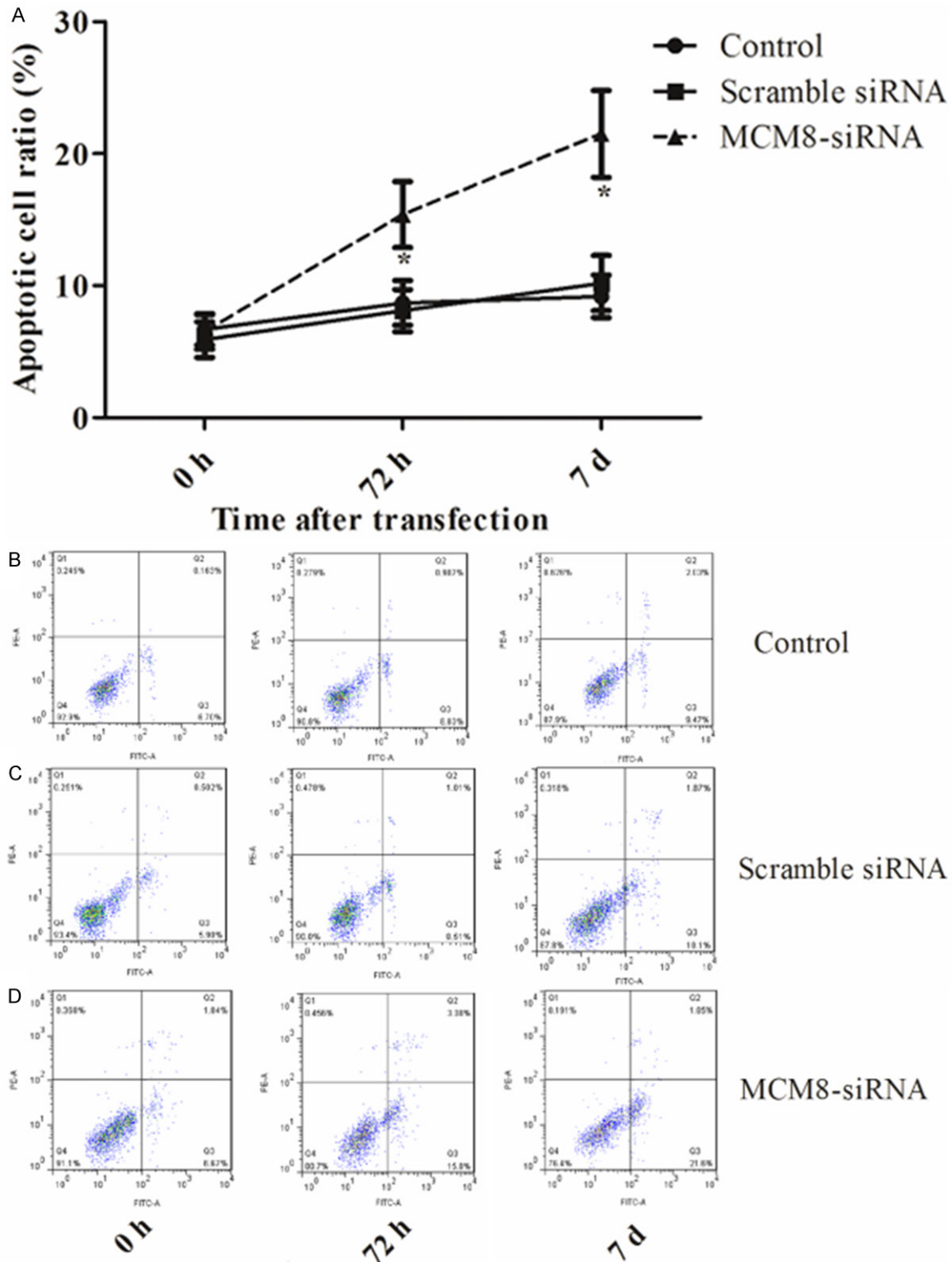


Figure 4. Apoptosis analyses after transfection with MCM8 siRNA. FCM assay is performed to measure apoptotic cells after transfection with MCM8 siRNA (at 0 h, 72 h and 7 d). A. Apoptotic cell ratio; B. Apoptotic cell at 0 h; C. Apoptotic cell at 72 h; D. Apoptotic cell at 7 d. MCM, Minichromosome maintenance proteins; FCM; flow cytometry; siRNA, small interfering RNA. * $P < 0.05$ compared to the control group or the scramble siRNA group.

addition, knockdown of MCM8 reduces the cell viability and while induces apoptosis in human

CML cell line K562. The induction of apoptosis might be through regulating the expression of

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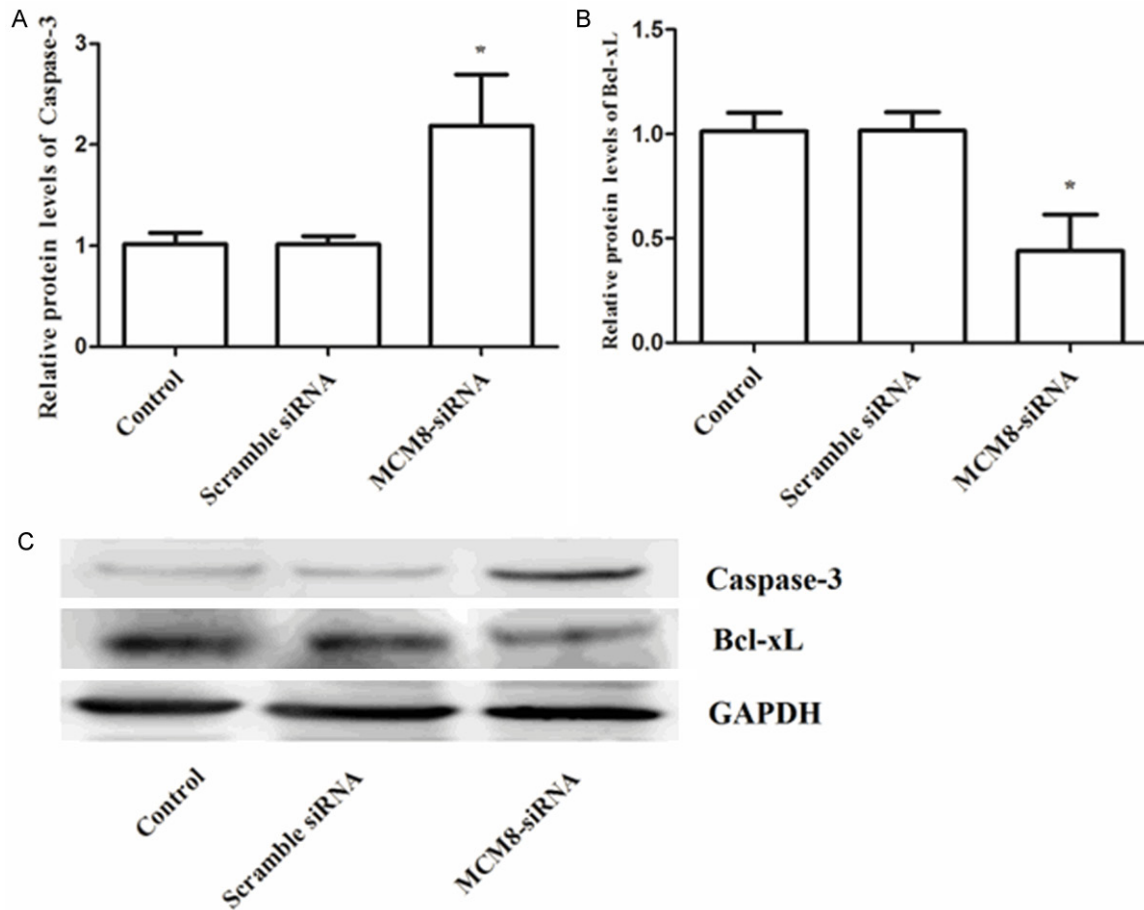


Figure 5. Expression of Caspase-3 and Bcl-xL. We analyze the expression levels of Caspase-3 and Bcl-xL after transfection with MCM8 siRNA using Western blotting. A. Relative protein levels of Caspase-3; B. Relative protein levels of Bcl-xL; C. Representative Western blotting picture of Caspase-3 and Bcl-xL protein in K562 cells. MCM, Minichromosome maintenance proteins; siRNA, small interfering RNA; Bcl, B-cell lymphoma. * $P < 0.05$ compared to the control group or the scramble siRNA group.

Caspase-3 and Bcl-xL. Therefore, the results suggest that MCM8 plays an important role in CML, and knockdown of MCM8 might be a potentially targeted therapy for CML.

DNA replication and DNA damage repair systems are essential factors in the tumor proliferation by preventing cancer through preservation of genome integrity [18, 19]. Oncogenic stimuli promote cell proliferation, in which DNA replication is required for the process. The DNA damage activates DNA damage checkpoint pathways, leading to cell cycle arrest, apoptosis or senescence to prevent the development of cancers [20]. Previous studies have indicated that DNA damage-response pathway is triggered in BM of chronic-phase CML patients possibly because of an enforced proliferation signal by BCR-ABL expression [21]. Besides,

cell-cycle checkpoints and DNA repair are critical for the survival, progression, and resistance of CML [22]. MCMs family appears the important factor for the control of DNA replication [23]. MCM8 is a new member of the MCM protein family and expressed in placenta, lung, liver, and heart. The gene includes 19 exons and located contrapodal to GCD10 at chromosome band 20p12.3-1 [24]. MCM8 is most like MCM7 in the region of potential helicase motifs. However, it is unlike MCM2-7 or MCM10 that are structure-bound in cells arrested in the late G1 phase [25]. It is structure-bound in the early S phase [15]. Also, the potential helicase domain of MCM8 is different from other MCM proteins. Given those factors, studies have suggested that MCM8 might interact with other MCM proteins to implement its functions. It has

reported that MCM8, associating with MCM4, MCM6 and MCM7 proteins, plays a significant role in DNA replication. But MCM8 may extend the range of MCM functions beyond replication. However, the exact function remains controversial. It has been acted as a component of pre-replication complexes interacting with CDC6 protein [26]. Another study, however, suggested that MCM8 functions as a DNA helicase during replication elongation [27]. In contrast to these studies, Blanton *et al.* suggested that the *Drosophila* MCM8 homolog mutants played significant role in meiosis but not in DNA replication [28]. Several studies showed that MCM8 was not as essential for DNA replication as the MCM2-7 complex but played a significant role in DNA repair [29-32]. Therefore, in view of its structure and functions, MCM8 has been recommended as a novel target for cancer researches. It has been well demonstrated that the MCM8 protein is abnormally expressed in colorectal cancer [17, 24], hepatocellular carcinoma (HCC) infected by Hepatitis B Virus (HBV) [33], choriocarcinoma [34], and uterine cervical cancer [25]. Therefore, we speculated that MCM8 might be involved in the pathogenesis of CML due to its DNA replication and DNA repair functions.

To confirm the hypothesis, we enrolled CML patients as well as healthy controls. The expression of MCM8 was compared between the patients. The results indicated that CML patients had higher levels of MCM8 either from PBMC or from BMMC, suggesting that abnormal activation of MCM8 during CML cell proliferation. A further functional mechanism of MCM8 in CML was elucidated in detail in K562 cell line. Using of siRNA technology to down-regulate MCM8 gene expression results in the decrease of cell proliferation but the increase of apoptosis. Further, induction of apoptosis was explored by determining the expression of apoptosis related proteins (Caspase-3 and Bcl-xL). It has been well demonstrated that anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL are frequently increased in tumors [35]. They prevent apoptosis by binding to Bax or Bak. Moreover, Caspases prominently contribute to the initiation (e.g. Caspase-8 and -9) and the execution (e.g. Caspase-3) of the apoptotic program [36]. Our data showed that knockdown of MCM8 significantly induced CML apoptosis by increasing pro-apoptotic protein

Caspase-3 but decreasing anti-apoptotic protein Bcl-xL. Thus, MCM8 may serve as a potential targeted therapy for the treatment of CML.

To summarize, our main finding is related to the significant role of an abnormal expression of the MCM8 protein in CML, and knockout MCM8 might have an effective therapy for CML. However, further study should be performed to elucidate the functions of MCM8 on CML.

Disclosure of conflict of interest

None.

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