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ORIGINAL ARTICLE

siRNA-mediated downregulation of BATF3 diminished proliferation and induced apoptosis through downregulating c-Myc expression in chronic myelogenous leukemia cells

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Abstract

Objective Despite considerable improvement in therapeutic approaches to chronic myeloid leukemia (CML) treatment, this malignancy is considered incurable due to resistance. However, investigating the molecular mechanism of CML may give rise to the development of extremely efficient targeted therapies that improve the prognosis of patients. Basic leucine zipper transcription factor ATF-like3 (BATF3), as transcription factor, is considered a key regulator of cellular activities and its function has been evaluated in tumor development and growth in several cancer types. This study aimed to evaluate the potential of the cellular impact of siRNA-mediated downregulation of BATF3 on CML cancer cells through cell proliferation, induction of apoptosis, and cell cycle distribution.

Materials and methods The transfection of BATF3 siRNA to K562 CML cells was performed by electroporation device. To measure cellular viability and apoptosis, MTT assay and Annexin V/PI staining were carried out, respectively. Also, cell cycle assay and flow cytometry instrument were applied to assess cell cycle distribution of K562 cells. For more validation, mRNA expression of correlated genes was relatively evaluated by quantitative real-time polymerase chain reaction (qRT-PCR).

Results The data indicated that siRNA-mediated BATF3 inactivating severely promoted the cell apoptosis. Also, the targeted therapy led to high expression of Caspase-3 gene and Bax/Bcl-2 ratio. Silenced BATF3 also induced cell cycle arrest in phase sub-G1 compared to control. Finally, a noticeable decrement was obtained in c-Myc gene expression through suppression of BATF3 in CML cells.

Conclusion The findings of this research illustrated the suppression of BATF3 as an effective targeted therapy strategy for CML.

Keywords Chronic myeloid leukemia · BATF3 · Cell proliferation · Apoptosis · c-Myc

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Introduction

Chronic myeloid leukemia, also called chronic myelogenous leukemia (CML), is considered a chronic and monoclonal myeloproliferative disease arising from the neoplastic alteration of the primary hemopoietic stem cell due to an acquired genetic defect. CML is the first neoplastic development related to constant acquired genetic dysfunction. Even now, it is considered the best evaluated molecular model for leukemia disorder. It is characterized by a particular chromosomal translocation between the long arms of chromosomes 9 and 22, t (9;22) (q34; q11.2) or Philadelphia chromosome, which gives rise to the fusion of a specific oncogene known as BCR-ABL1. This oncoprotein can stimulate various regulatory signaling pathways that result in cellular proliferation without any regulation of cytokines and the effect on bone marrow stroma [1]. CML involves the lineage of myeloid, erythroid, megakaryocytes, monocyte, B cells, and sometimes T cells, but it indicates no effect on bone marrow stromal cells [2]. CML is considered rare cancer responsible for up to 15% of leukemia patients with a rate of l-2 cases per 100,000 population and is reported to be higher in men than females [3, 4]. Heterogeneity among patients with leukemia and even among leukemia cells has become a major challenge in the treatment methods [5]. In monitoring CML disorder, numerous therapy methods have been applied, such as three tyrosine kinase inhibitors (TKIs), nilotinib, dasatinib, and imatinib, that are approved as a first-line cure in patients with chronic phase (CML-CP) [6]. However, because of drug resistance, the majority of patients have no effective response to drug therapy and face recurrence. Recently, bone marrow transplantations have been presented as a novel method for CML, that is accompanied by a high risk of morbidity and mortality [7]. Hence, practical strategies such as targeted therapy that can target molecular mechanisms may elucidate new treatment approaches for treating CML patients [8].

The numerous lymphoid lineages are primarily modulated by a number of transcription factors, such as the dimerizing basic leucine zipper (bZIP) proteins, identified as activator protein 1 (AP-1) [9]. AP-1 has important functions in cellular proliferation, differentiation, and apoptosis, and dysregulation of AP-1 is considered a feature of various pathologies, especially cancer [10]. The BATF family contains BATF, BATF2, and BATF3, belonging to the family of bZIP transcription factors, and consists of an alpha-helical bZIP domain containing a DNA-binding domain and a leucine zipper motif without a transactivation domain. Primarily, this family was thought to be negative regulators of AP-1 driven transcription because competing with Fos for cooperation with Jun resulted in heterodimers and generating bZIP dimers that prevent the transcription of AP-1 reporter genes [11]. However, recently, it has been reported that these factors have a positive transcriptional interaction with the interferon-regulatory factor family members and adjust numerous characteristics of B and T cell function, which are required for cellular responses. Besides, all three BATFs indicate compensative functions with each other in numerous immune cell lineages [12].

BATF3, as a member of the AP-1 family, has a normal expression in a type of T cells, namely T helper1, as well as in conventional dendritic cells (cDCs). BATF3 is usually not expressed in normal B-cells excepting CD30-positive B-cells of reactive lymph nodes [13]. BATF3 indicates the important function in the CD8α$^+$ classical DCs developing in lymphoid tissues, which prime CD8$^+$ T cell reactions through cross-presentation [12] to manage infection of intracellular pathogens. Actually, mice with BAFT3-deficient have no ability to cross-present antigens that may be susceptible to specific viral infections and tumorigenesis. This might suggest that BATF3 is likely upregulated in oncogenic conditions [14]. Also, it has been reported that the human B-ATF gene is highly expressed in hematopoietic tissues [11]. Accordingly, considering the important role of BATF3 in cancer progression, the current research aimed to evaluate the effect of BATF3 suppression on the CML cell viability and apoptosis capacity.

Materials and methods

Cell culture and transfection

The human CML cell line of K562 was obtained from the Pasteur Institute (Tehran, Iran) and stored in a liquid nitrogen tank. After defrosting, cells were cultivated in T25 flasks containing RPMI1640 medium enriched with 10% FBS (Gibco, USA), 4 µm L-glutamine, 100 U/ml penicillin, and 100 µg/ml Streptomycin (Gibco, USA). Subsequently, the K562 cells were maintained in an incubator with 95% humidity and 5% carbon dioxide. After several times of sub-culturing the cells to enter the logarithmic phases, 5×10^5cells/ml were transfected with BATF3-siRNA at different concentrations (60, 80 and 100 pmol) and times (24, 48, and 72 h) using Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, California, USA) according to the manufacturer's protocols (cuvettes: 0.4 cm^3; time constant: 12.5 ms; voltage: 160 v). The optimum dose and time were selected for further experiments. The documented sequence of BATF3-siRNA is listed in Table 1.

Table 1 The sequence of siRNA designed for BATF3

siRNA	Sense	Antisense
BATF3 siRNA	5´-CUCUAGAAUUUGGAUAAUAAAGATG-3´	5´-CAUCUUUAUUAUCCAAAUUCUAGAGGA-3´

Quantitative real-time PCR analysis (qRT-PCR)

To validate the silenced BATF3 expression at the RNA level and evaluate expression levels of related genes in K652 cell line, qRT-PCR was performed. Cellular RNA was extracted based on the approaches approved by the Trizol RNA extraction kit (GeneAll, Korea). The pureness and concentration of extracted RNA were measured via NanoDrop2000 (Thermo Scientific, USA). Subsequently, 1 µg RNA of each sample was subjected to cDNA synthesis (BIOFACT, Korea) using a thermal cycler system (Bio-Rad, Hercules, CA) according to the procedures provided by the manufacturer. The expression levels of BATF3, Caspase-3, Bax, Bcl-2, and c-Myc genes were evaluated by the BioFACT™ 2X Real-Time PCR Master Mix (Korea) in a light cycler system (Roche Diagnostics, Mannheim, Germany). To measure the relative gene expression of related genes, the comparative $2^{-\Delta\Delta CT}$ method was utilized. The sequences of primers used in this study are presented in Table 2.

MTT Assay

Cellular proliferation was investigated by MTT assays (Sigma-Aldrich, USA). K562 cells were transfected with BATF3 siRNA and in a density of 5×10^4 cells/ml cultured in 96-well plates in three groups, including BATF3 siRNA, Scrambled siRNA (negative control), and control incubated for 48 h. A total of 50 µL MTT solution was added to each well, and the plate was placed in an incubator for 3–4 h. To dissolve formazan crystals, 100 µL of DMSO (dimethyl sulfoxide) was added to each well. After that, the plate was subjected to an ELISA reader (Sunrise RC, Tecan, Switzerland) to determine the optical density of each well at the wavelength of 570 nm.

Apoptosis assay

To evaluate the apoptosis-mediated cell death in transfected and control groups, flow cytometry analysis (MiltenyBiotec™ FACS Quant10; MiltenyBiotec, Germany) using Annexin V/PI staining was performed. K562 cells were transfected with BATF3 siRNA and, at a density of 10×10^5 cells/well, were seeded into 6-well plates. Th cells were incubated for 48 h, and then harvested by centrifuging at 500 rpm for 5 min at 4 °C. In the next step, samples were washed with PBS. After removal of PBS and dispersing the cell pellets, they were incubated by annexin V (5 µL), propidium iodide (5 µL), and binding buffer (200 µL) for 15 min on ice in a dark condition. The stained cells were washed with PBS again and then subjected to flow cytometry system. The obtained data were analysed using FlowJO version 10 software (FlowJO LLC., Ashland, OR, USA).

Cell cycle analysis

The status of cell cycle progression in samples through BATF3 suppression was investigated using the PI intracellular staining and flow cytometry analysis. After 48 h of transfection, the cells were collected and washed with PBS. After that, ethanol (70%) was added to the cells for the proper fixation before the permeabilization. After maintaining at − 20 °C for 24 h, we added 1 mg/ml RNase A (Bioneer, Daejeon) to the samples and incubated them for 30 min. Then, the cells were immersed in a 500 µL PBS solution containing 1 ml of 10 µg/ml of PI and 0.1% triton x100 following the manufacturer's instructions. After 10 min of incubation in the darkness, the cells were subjected to MACSQuant flow cytometry to clarify DNA content and the cell cycle distribution. FlowJo software was used to analyze the percentage of the cells in each phase of cell cycle.

Table 2 The sequences of expression primers used in the study

Target	Forward primer (5'-3')	Reverse primer (5'-3')
BATF3	CTGAGGATGATGACAGGAAGG	GCAGCATGGTGTTTTCTTGC
Bcl-2	GAGTTCGGTGGGGTCATGTG	CACCTACCCAGCCTCCGTTA
Caspase-3	CAAACCTCAGGGAAACATTCAG	CACACAAACAAAACTGCTCC
Bax	TTTGCTTCAGGGTTTCATCCA	TCTGCAGCTCCATGTTACTGTC
c-Myc	CACATCAGCACAACTACGCA	GCTCCAAGACGTTGTGTGT
GAPDH	AACATCATCCCTGCCTCTAC	CTGCTTCACCACCTTCTTG

Statistical analysis

The results were expressed as mean values ± standard deviation (SD). Student's t-test and one-way analysis of variance (ANOVA) were used for statistical analysis between two and more than two groups using GraphPad Prism version 7.0 software (San Diego, USA). A p-value less than 0.05 was considered as statistically significant.

Results

BATF3 knockdown suppressed the proliferation of K562 cells

To suppress *BATF3* expression in K562 cells, specific siRNA targeting *BATF3* was transfected into these cells. qPCR results showed that different amounts of *BATF3* siRNA significantly and stably decreased *BATF3* mRNA expression till 48 h after transfection compared to un-transfected cells and scrambled siRNA transfected cells (Fig. 1A and B).

Besides, as illustrated in Fig. 2, MTT assay revealed that suppressing *BATF3* expression could significantly ($p < 0.0001$) diminish K562 cell proliferation to 78.29 ± 2.955% in comparison with control (100 ± 1.204) and scrambled siRNA (96.30 ± 1.935) groups. Thus, it was suggested that BATF3 might function as a crucial transcription factor involved in CML cell proliferation and growth.

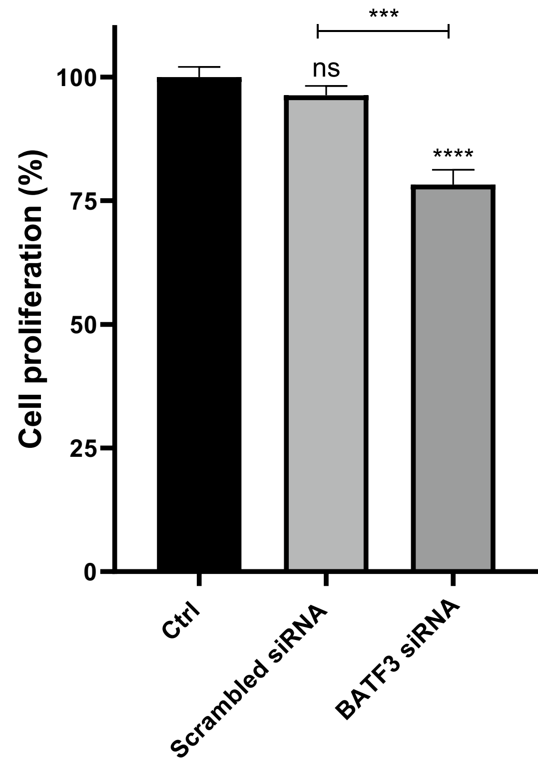

Fig. 2 MTT assay results. BATF3 suppression significantly decreased the proliferation of K562 cells; *** p < 0.001and ****. p < 0.0001

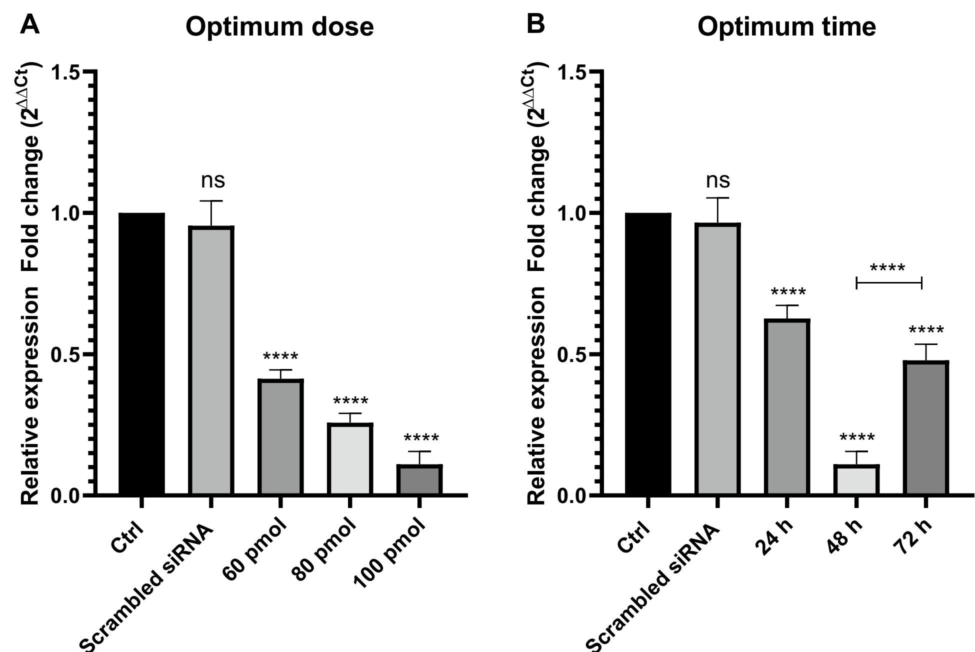

Fig. 1 qPCR results show the suppression of BATF3 mRNA expression in K562 cells; ****p < 0.0001

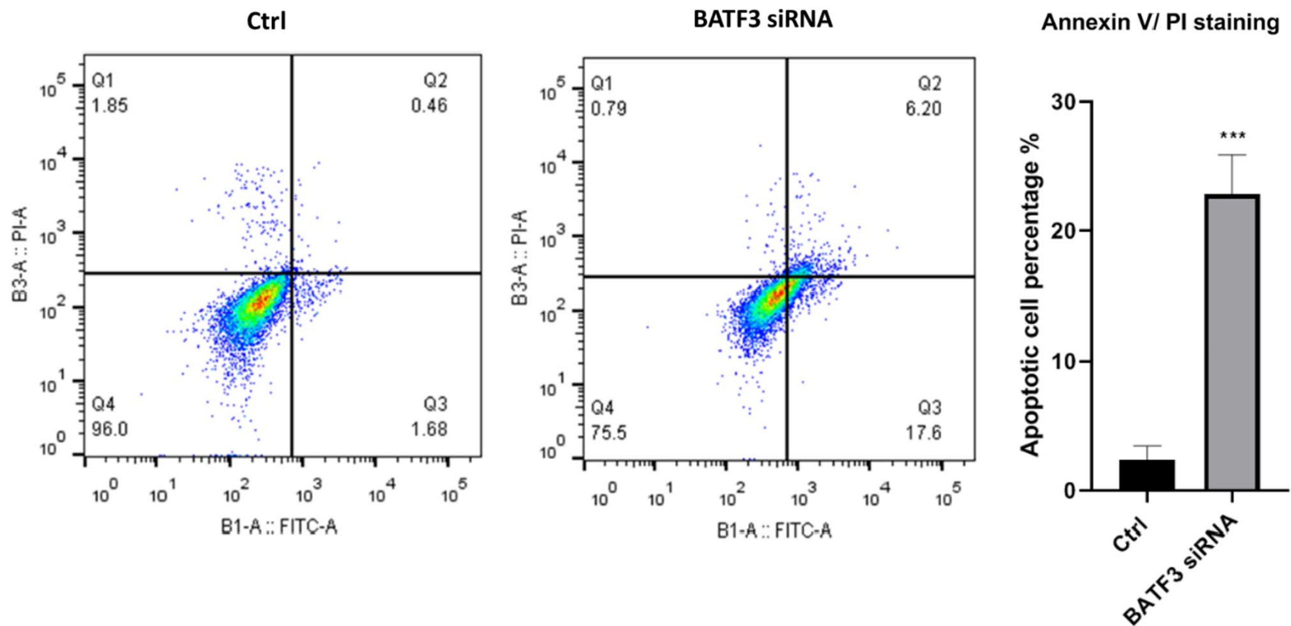

Fig. 3 Annexin V/PI results. K562 CML cells underwent cell apoptosis after transfection with BATF3 siRNA; ***p < 0.001

Suppression of BATF3 induced programmed cell death in K562 cells

To illustrate the effectiveness of *BATF3* suppression on K562 CML cells, FITC-annexin V/PI staining was carried out. As depicted in Fig. 3, the reduced expression of *BATF3* resulted in a rise in the early apoptotic cell percentage (FITC$^+$, PI$^-$) by 17.6% and late apoptotic cell percentage (FITC$^+$, PI$^+$) by 6.20% in comparison with the control cells, indicating 1.68% early apoptosis and 0.46% late apoptosis. Collectively, these results implied that decreasing the expression of BATF3 in K562 CML cells could considerably induce cell apoptosis ($p < 0.0001$).

Subsequently, qPCR was employed to evaluate the expression of apoptosis regulators in treatment groups. The obtained results further evidenced that *BATF3* suppression led to significant upregulation of *Bax* ($p < 0.0001$) and *Caspase* 3 ($p < 0.0001$) gene expression in K562 cells. Also,

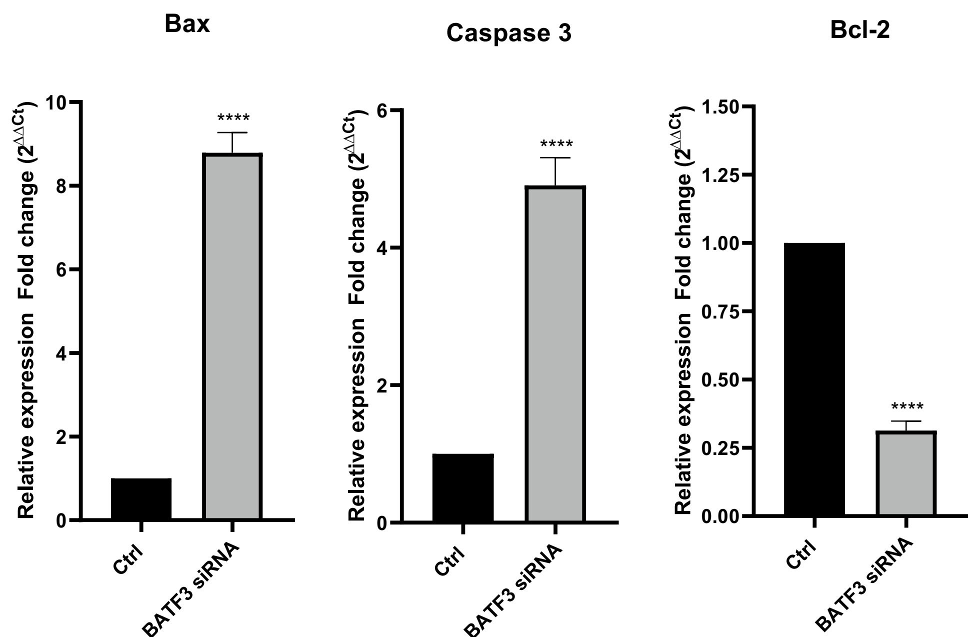

Fig. 4 QRT-PCR results. The graphs show the changes in the expression levels of apoptosis-related genes, *Bax*, *Caspase 3,* and*Bcl-2* in K562 cells in mRNA levels after transfection with *BATF3* siRNA; ****p<0.0001

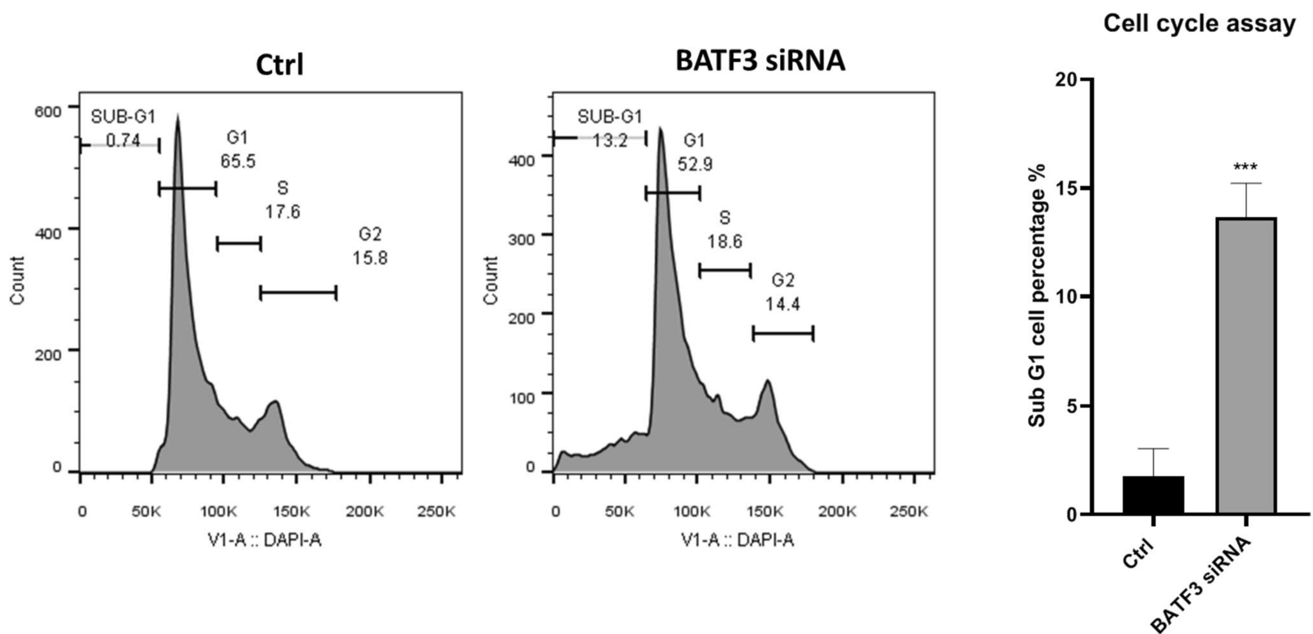

Fig. 5 Flow cytometry analysis of cell cycle status in treatment groups. *BATF3* knockdown induced sub G1 cell cycle arrest in K562 cells; ****p<0.001*

Bcl-2 survival gene expression was significantly downregulated ($p < 0.0001$) after transfection of the cells with *BATF3* siRNA (Fig. 4).

Sub G1 cell cycle arrest was induced by suppression of BATF3 in K562 cells

The role of BATF3 was also investigated in regulating the cell cycle process in K562 cells. As shown in Fig. 5, flow cytometry analysis using PI staining indicated that downregulation of *BATF3* increased the portion of cells accumulated at the sub-G1 phase from 0.74% in control cells to 13.2% in the BATF3 siRNA-transfected group ($p < 0.0001$). This result further confirmed the anti-apoptotic function of BATF3 in CML cells. Nonetheless, no significant increase was evidenced in the percentage of cells in G1, S, and G2 phases after transfecting the cells with BATF3 siRNA.

BATF3 suppression led to downregulation of *c-Myc* oncogene

The transcription factor of c-Myc is recognized as an imperative oncogene regulated by BATF3 and participates in cell cycle regulation. Besides, CML progression has been linked with Bcr-Abl-induced c-Myc expression. Hence, this study also aimed to examine the effect of *BATF3* on this aspect of CML tumorigenesis. Interestingly, qPCR results showed that *BATF3* downregulation using specific siRNA caused a remarkable decrease ($p < 0.01$) in the mRNA expression levels of *c-Myc* in K562 cells (Fig. 6). This finding illustrates the therapeutic effect of BATF3 through regulating the activity of c-Myc in CML cells.

Discussion

CML is one the most common myeloproliferative neoplasm, which is mainly caused by the translocation BCR-ABL [14]. This event leads to the production of fusion protein of Bcr-Abl, a tyrosine kinase that is constitutively activated through tumorigenesis, causing uncontrolled CML cell proliferation. Subsequently, TKIs, such as imatinib, are considered as the first treatment options for this malignancy [15]. Despite the improvement of patients' survival rates over the decade, there has been an increase in TKI resistance in CML patients, which makes this leukemia incurable in some cases [16]. Following treatment options for resistant patients, stem cell transplant therapy is also restrained by the availability of donors [17]. These facts illustrate that the identification of molecular pathways involved in CML incidence and progression is a constant need to develop new therapeutic strategies to overcome such obstacles in the improvement of CML patients' survival.

Of interest, BATF3, which functions as a transcription factor, has attracted scientists' attention as a promising therapeutic target for human cancers. BATF3 is highly expressed in conventional Cd11c-positive dendritic cells [18] and it is involved in the homeostatic development of

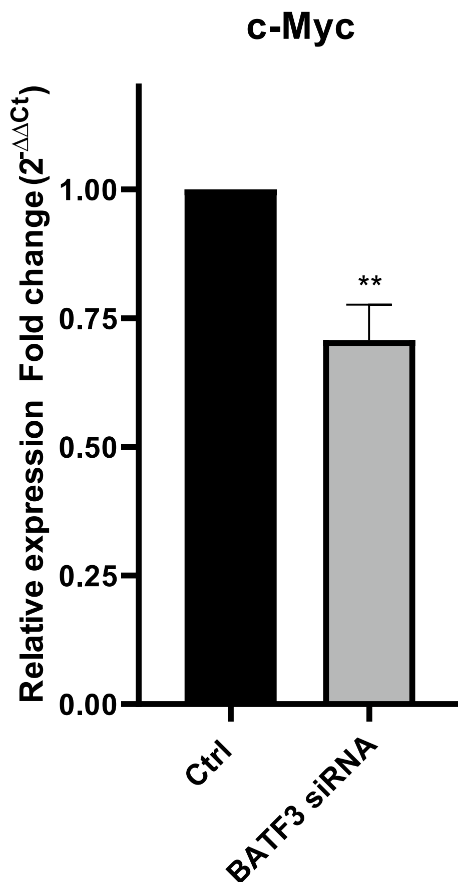

Fig. 6 QRT-PCR results showing *c-Myc* mRNA expression. The suppression of *BATF3* significantly downregulated *c-Myc* expression; **$p<0.01$

CD8-alpha-positive dendritic cells that trigger the responses of CD8 T-cell against intracellular pathogens [19]. However, BATF3 dysregulation also plays an essential role in the initiation and progression of different types of human cancer. It has been shown that BATF3 exhibits high expression levels in colorectal cancer tissue and cells, promoting in vivo and in vitro tumor growth and invasion by regulating S1PR1/p-STAT3/miR-155-3p/WDR82 axis and AP-1/cyclinD1 signaling, as well as involving in the PD-L1 induced immune evasion of colorectal cancer cells [20, 21]. Interestingly, in a feedforward signaling loop, BATF3 has been recently identified to participate in Hodgkin lymphoma development by upregulating the S1PR1 and S1P pathways [22].

Our results also evidenced that BATF3 may function as a key transcription factor involved in regulating CML cell proliferation. It was shown that suppressing BATF3 expression using specific siRNA reduced K562 CML cell proliferation through apoptosis induction. Furthermore, BATF3 knockdown was evidenced to increase the sub-G1 phase cell cycle arrest, indicating this transcription factor's role through CML tumorigenesis and progression. Consistence with these results, previous studies illustrated the regulation of in vitro cell proliferation, invasion, and migration of colorectal cancer cells through upregulation of the expression of S1PR1. In turn, S1PR1 promotes malignant features in these cells through increasing the activity of p-STAT3. Besides, the suppression of BATF3/S1PR1/p-STAT3 pathway was shown to diminish in vivo CRC tumor growth [21]. In our study, qPCR results showed that BATF3 regulates Bax and Bcl-2 expression. Its suppression led to Bax upregulation and Bcl-2 downregulation in CML cells, further illustrating the BATF3 involvement in apoptosis regulation. The low *BAX/BCL-XL* expression ratio showed a negative correlation with BCR-ABL/ABL expression, correlating with the progression of malignancy and poor prognosis of patients. TKIs, as one of the treatment options for CML, have also been illustrated to reduce BAX*/BCL-X* in patients and CML cells [23]. Besides, Bcl-2, an anti-apoptotic agent, functions as a key regulator in the survival of CML stem cells, and targeting this pro-survival gene is considered a promising strategy in combination with BCR-ABL tyrosine kinase-based therapies that improve the outcomes of patients [24]. Our study also implied that *BATF3* suppression in CML cells led to overexpression of caspase 3, as an effector caspase that interacts with caspase 8 and caspase 9 and induces programmed cell death [25, 26]. Furthermore, p53 expression has been shown to increase the activity of caspases, including caspase 3, which is involved in BCR-ABL and C-ABL cleavage, as the most significant effectors in leukemogenesis. Subsequently, this event provokes the erythroid differentiation in K562 CML cells [27, 28]. Therefore, BATF3 could be suggested as a promising target for CML, considering its involvement in CML cell apoptosis through regulating the mentioned apoptosis major regulators.

As one of the oncogenic transcription factors participating in the majority of human cancers, c-Myc plays a significant role in the regulation of hematopoietic cell proliferation, differentiation apoptosis, and tumorigenesis [29]. Also, c-Myc is considered an important effector in oncogenic transformation in CML, which is overexpressed in mRNA and protein levels through Bcr-Abl tyrosine kinase-dependent activity of JAK2 signaling [30].

Interestingly, Imatinib tyrosine kinase inhibitor has been evidenced to exert its therapeutic effects on CML cells through downregulation of c-Myc expression. In contrast, the high expression of c-Myc in CML patients, through switching the chronic phase to blast crisis, plays an indispensable role in the appearance of resistance to TKI inhibitors, including imatinib [31]. Targeting c-Myc is suggested as a useful alternative therapeutic strategy for overcoming CML poor outcomes. Our study illustrated that BATF3 suppression in CML cells significantly downregulated c-Myc, highlighting another aspect of the therapeutic importance of BATF3 for this type of leukemia.

Besides, BATF3 has been revealed to bind the MYC promoter, and its overexpression is mediated by JAK/STAT signaling to provoke the activity of MYC in classical anaplastic large cell and Hodgkin lymphomas [32].

Although the results obtained from this study are very promising due to specific gene silencing, transfection of siRNA into cells has limitations. One of the obstacles to the successful administration of siRNA in vivo is the degradation by enzymes present in tissue and serum. Considering that the half-life of naked siRNAs in the serum varies from a few minutes to a few hours, their accumulation in the desired location is considered a big challenge [33]. In addition, siRNA can have unwanted off-target effects because nucleotides 2–8 of an siRNA may repress gene expression by pairing with unrelated mRNAs. In some cases, this off-target effect can reduce protein levels almost as much as the effect of siRNA on the desired target [34]. Many studies have shown that different characteristics of the sequence, structure, and mode of delivery of siRNA can stimulate the immune response and cause adverse immunological effects [35]. It is hoped that further studies can overcome these limitations.

Conclusion

Our findings implied the significance of BATF3 in CML progression by regulating cell proliferation and apoptosis. BATF3 was illustrated to exert its effect on CML cells by regulating apoptosis and survival-related genes, such as *Caspase 3, Bax* and *Bcl-2*. Besides, suppression of BATF3 led to the downregulation of c-Myc oncogene, as an important regulator of cell cycle and cell proliferation in K562 CML cells, indicating the therapeutic importance of BATF3 for CML. However, these results need to be validated by further functional analysis and using in vivo experiments and clinical trials.

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Authors' contributions RD: Conceptualization, investigation, formal analysis, Writing—Original Draft. VKS: Validation, formal analysis, data curation. SS: Validation, formal analysis, data curation. MA: Validation, formal analysis, data curation. SMBT: Writing—review & editing, data curation. DS: Writing—review & editing, data curation. ORF: Validation. EM: Supervision, project administration. BB : Supervision, project administration.

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Data availability All data generated or analyzed during this study are avaidable upon request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement All experiments and procedures were conducted in compliance with the ethical principles of Shiraz University of Medical Science, Shiraz, Iran and approved by the regional ethical committee for medical research.

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