

***Bcr-abl* Silencing by Specific Small-Interference RNA Expression Vector as a Potential Treatment for Chronic Myeloid Leukemia**

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ABSTRACT

Background: RNA interference (RNAi) is the mechanism of gene silencing-mediated messenger RNA degradation by small interference RNA (siRNA), which becomes a powerful tool for *in vivo* research, especially in the areas of cancer. In this research, the potential use of an expression vector as a specific siRNA producing tool for silencing of Bcr-abl in K562 cell line has been investigated. **Methods:** siRNA specific for Bcr-abl as short hairpin RNA (shRNA) was designed and cloned in expression vector (pRNAH1.1/Neo). K562 cells were cultured in RPMI media and transfected with shRNA expressing vector using lipofectamin 2000. Successful transfection was confirmed by significant increase of enhanced green fluorescent protein (EGFP) levels in K562-treated cells with expression vector (pEGFP-C1). *In vitro* studies in human K562 cell line entailed modulation of endogenous Bcr-abl mRNA levels which induced apoptosis. Effects of siRNA treatment on K562 cells were measured by ELISA. **Results:** Successful expression of siRNA was confirmed by significant reduction of Bcr-abl mRNA levels in K562 cells treated with expression vector (pRNAH1.1/Neo). siRNA directed against Bcr-abl effectively induced apoptosis and reduced viability in human K562 cell lines. **Conclusion:** Expression vector of siRNA can be used *in vitro* to target specific RNA and to reduce the levels of the specific gene product in the targeted cells. Results of this work suggest that RNAi has potential application for the treatment of a variety of diseases, including those involving abnormal gene expression and viral contamination. *Iran. Biomed. J. 14 (1 & 2): 1-8, 2010*

Keywords: Apoptosis, SiRNA, K562 cell line, Bcr-abl

INTRODUCTION

RNA interference (RNAi) is a regulatory mechanism which is highly conserved throughout many eukaryotic organisms, in which mediates sequence-specific post-transcriptional gene silencing by the presence of double-stranded RNA (dsRNA) in a cell, which causes the destruction of mRNA with sequence homology to the dsRNA [1-3]. This phenomenon presents high importance for therapeutic applications. Elbashir *et al.* [4] have shown that synthetic RNA (21-23 bp) are able to mediate

cleavage of the target RNA and called small interfering RNA (siRNA). Recently, it has been demonstrated that RNAi mediated by siRNA can specifically target sequences from different oncogenes and viruses [5-8]. Because exogenous application of siRNA can efficiently trigger RNAi in mammalian cells [9], siRNA are increasingly used in transient (co)transfection assays to modulate gene expression in mammalian cells, including human cells [10, 11]. There are two possible approaches, chemically synthetic siRNA and vector-based expression, for modulating gene expression in mammalian cells. The chemically synthetic siRNA

*Corresponding Author; Tel & Fax: (+98-21) 2283 0262; E-mail: javadihr83@yahoo.com; **Abbreviations:** RNA interference (RNAi), small interference RNA (siRNA), short hairpin RNA (shRNA), double-stranded RNA (dsRNA), chronic myeloid leukemia (CML), Reverse transcription-PCR (RT-PCR), double distilled water (DDW), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), single stranded DNA(ssDNA)

has a short half-life and is needed to transfect frequently [12-13]. Using a suitable siRNA expression vector, the siRNA level will increase for a longer time than chemically synthetic siRNA. The leukemic cells in approximately 95% of chronic myeloid leukemia (CML) and 25% of acute lymphoblastic leukemia contain a characteristic balanced translocation, t(9;22) (q34;q11), manifesting as an abnormal Philadelphia chromosome. This translocation results in the fusion of a portion of the *Bcr* gene to the *abl* gene and generates the *Bcr-abl* fusion gene [14-16]. The Philadelphia chromosome encodes a constitutively active cytoplasmic tyrosine kinase, which is believed to be an early event which is necessary and sufficient to induce and maintain leukemic transformation [17]. Recently, the development of molecularly targeted leukemia therapies has resulted in the clinical use of a small molecule such as imatinib mesylate. This small molecule inhibits the tyrosine kinase catalytic domains of Bcr-abl and several other kinases [18]. Treatment with imatinib has resulted in a complete cytogenetic remission in the majority of CML patients; however, many patients have shown imatinib resistance. The mechanism of resistance to imatinib ranges from nonspecific multidrug resistance [19] including overexpression of Bcr-abl due to gene amplification [20] and point mutations, in the ABL kinase domain [21]. Point mutations, which impair imatinib binding by interrupting the critical contact point or by inducing a conformation to which imatinib binding is reduced [22]. Fusion-transcripts encoding oncogenic proteins may represent potential targets for a tumor-specific RNAi approach. Therefore, in the patients who do not respond to imatinib, a highly specific Bcr-abl targeting approach would be the clinical use of RNAi. By silencing of Bcr-abl using a specific siRNA, the cytoplasmic tyrosine kinase activity reduces and induces apoptosis. Thus, Bcr-abl silencing using siRNA might be used as a new therapy for CML.

The aim of this study was induction of apoptosis in K562 cell line by Bcr-abl silencing using an expression siRNA vector (pRNAH1.1/Neo). It has been demonstrated that the expression vector cloned with the anti-Bcr-abl expression DNA specifically inhibits Bcr-abl mRNA expression and induces apoptosis. Therefore, this approach of gene silencing may lead to RNAi-based therapeutics.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. K562 (human CML, NCBI-C122) cell line was obtained from Pasture Institute of Iran (Tehran). RPMI 1640 medium, fetal calf serum, L-glutamine, lipofectamine™ 2000, streptomycin sulphate, penicillin G sodium were purchased from Invitrogen (Paisley, UK). Expression siRNA vector (pRNAH1.1/Neo, Genscript Co.), HEPES, MgOAc, BamHI, HindIII, T4 ligase, Tango buffer [33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA], dNTP ribonuclease inhibitor, RevertAid M-MuLV reverse transcriptase (RT), DNA extraction kit were procured from Fermentas Co. GFP expression vector (pEGFP-C1) (Clontech, US) and RNeasy columns were purchased from Qiagen (Basel, Switzerland) and cell death detection kit from Roche Applied Science Co. (Germany).

Cell culture. K562 cells were passaged twice weekly using RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, 100 µg ml⁻¹ streptomycin sulphate and 100 units ml⁻¹ penicillin G sodium. Then, they were kept in a humidified atmosphere of 5% CO₂ at 37°C [23-25]. Prior to transfection with expression siRNA vector (pRNAH1.1/Neo), RPMI 1640 medium were replaced by DMEM. A volume of cell suspension containing 4 × 10⁶ cells (0.4 ml) was dispensed into 12-well plates for transfection with expression siRNA vector (pRNAH1.1/Neo). Cell viability was measured by trypan blue exclusion assay, following standard procedures.

Small hairpin expression vector (pRNAH1.1/Neo) cloning. Twenty-one-nucleotide single-stranded RNA directed against the b3a2 fusion sequence of Bcr-abl gene (5'-AGUU CAAAAGCCCUUCAGCGG-3') were designed as short hairpin RNA (shRNA) 5'-CGC-GGA-UCC-CGC-GCU-GAA-GGG-CUU-UUG-AA C-UCU-UGA-UAU-CCG-GAG-UUC-AAA-AGC-C CU-UCA-GCG-UUU-UUU-CCA-AAA-GCU-UCG-C-3') according to Elbashir *et al.* [26] and were synthesized as ssDNA by Cinnagen Co. (Tehran, Iran). The control siRNA was composed of the scrambled b3a2 sequence (5'-CUUCCCGAAAACU UGAGACdTdT) not homologous to any human mRNA. Control siRNA comprised duplexes of 21 base pairs with two 3' deoxythymidine overhangs.

ssDNA was dissolved in annealing buffer (3M NaCl, 0.3M Sodium Citrate, pH:7) and double distilled water (DDW) was added to a final concentration of 6 mM and incubated at 90°C for 10 min, then slowly cooled. pRNAH1.1/Neo vector (20 µl = 4 ng) was incubated with 2 µl of *Bam*HI and *Hind*III in 5 µl of Tango buffer in 37°C for 4 h and digested vector was purified using DNA extraction kit. Digestion of the hybridized insert DNA was performed using the same method, but with incubation time of 12 h at 37°C and restriction enzymes were inactivated by incubation at 80°C for 20 min. The digested vector and insert DNA were ligated by addition of 10 µl of vector to 40 µl of insert DNA (40 ng/µl) in T4 specific buffer (6 µl) and T4 ligase (3 µl). The reaction solution was incubated at 10°C overnight. The T4 ligase was inactivated by heating at 65°C for 10 min.

Transfection of K562 cells. K562 cells were cultured as previously described [23-25]. Cells (4×10^6) were cultured in 12-well plates with 500 µl of DMEM medium free of FBS and antibiotic. pRNAH1.1/Neo vector (0.8 µg) and lipofectaminTM 2000 (2 µl) were dissolved in 100 µl of DMEM and left at RT for 5 min. The solution (100 µl) was added to K562 cells and incubated at 37°C and 5% CO₂ for 4 h. The DMEM media were replaced by RPMI containing 10% FBS and antibiotic and then incubated in 5% CO₂ at 37°C for 24 h. Transfected cells with mismatch siRNA, pRNAH1.1/Neo vector, GFP expression vector (pEGFP-C1) and lipofectaminTM2000 were used as control. The other transfection methods such as CaCl₂ and electroporation were tested and compared with lipofectaminTM 2000.

Fluorescence microscopy. To quantify transfection efficacy, green fluorescence of GFP protein in transfected cells by GFP expression vector (pEGFP-C1) was investigated using a fluorescence microscope.

Reverse transcription-polymerase chain reaction (RT-PCR) and PCR. Total RNA was extracted from K562 cells using RNAsy columns. RNA yield and purity were determined spectrophotometrically at 260/280 nm, and RNA integrity was verified using agarose gel electrophoresis. RT-PCR was performed as described before [27]. Briefly, solution reaction (total RNA, 300 ng; oligo(dT) primer, 1.5 µl and DDW, 6 µl) was incubated at 70°C for 5 min and immediately transferred on ice. Then, 4 µl reaction

RT-buffer, 2 µl dNTP 10 mM, 0.5 µl ribonuclease inhibitor and 12.5 µl DDW were added and incubated at 37°C for 5 min. Finally, 200 U RevertAid M-MuLV RT was added and heated at 42°C for 60 min. The RT-PCR product was amplified using PCR (solution reaction, dNTP [10 mM], 0.5 µl; MgCl₂ [25 mM], 1.25 µl; PCR buffer 10×, 2.5 µl; Taq DNA polymerase, 0.5 µl; cDNA, 2.5 µl; R-primer [10 pmole], 1.25 µl; F-primer [10 pmole], 1.25 µl and DDW, 15.25 µl) with specific primers for Bcr-*abl* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follow:

Bcr-*abl* forward primer:

3' -AGA-AGT-GTT-TCA-GAA-GCT-TCT-5'

Bcr-*abl* reverse primer:

5' -AGC-ACG-GAC-AGA-CTC-ATG-GG-3'
covering the exon b3/a2

GAPDH-forward primer:

3' - CAA-CAG-GGT-GGT-GGA-CCT-C-5'

GAPDH-reverse primer:

5' - TGG-TGG-TCC-AGG-GGT-CTT-A-3'

Apoptosis assay. The apoptosis induction was determined using apoptosis detection kit [28]. Briefly, 24, 48 and 72 h after siRNA treatment, 4×10^5 cells were washed in PBS and resuspended in 200 µL lysing buffer. Following shacking at 15-25°C for 30 minutes, lysate cells were centrifuged at 200 ×g for 10 min and 10 µl of supernatant was employed for ELISA analysis. The assay is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This follows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. The samples are placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. After removal of the unbound antibodies, the amount of peroxidase retained in immunocomplex is photometrically determined with (2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) as the substrate at 405 nm.

Statistical analysis. Results were expressed as mean ± SE. Differences between treatments with siRNA and the respective treatments with lipofectamin, mismatch siRNA, pRNAH1.1/Neo vector and pEGFP-C1 vector were analyzed using a two-tailed paired Student's *t*-test (StatView for PC). Significance was defined as $P < 0.05$.

Bcr-*abl* mRNA declined by approximately 30% (Fig. 4). Again, cultures of k562 cells treated with b3a2-1 resumed growth after 10 to 12 days in all experiments.

Table 1. Effects of anti-Bcr-*abl* siRNA on Bcr-*abl* mRNA expression and GAPDH mRNA as a internal control in k562 cells (n = 3).

	24 h	48 h	72 h
Control vector	101% ± 5.3	98.1% ± 4.4	99.5% ± 7
SiControl	91.7% ± 6.8	99% ± 5.8	102.3% ± 8.1
b3a2-1	42.2% ± 4.0*	35% ± 3.9**	30.2 ± 4.3**
GAPDH	100%	100%	100%

* $P < 0.05$, ** $P < 0.01$

Induction of apoptosis on Bcr-*abl* silencing. Bcr-*abl* has been reported to increase apoptosis resistance. As shown in Figure 5A, specific treatment with vector-based express siRNA against Bcr-*abl* leads to an increased apoptosis. Figure 5A shows an increase in the apoptotic cell population only when treated with sib3a2-1. Nevertheless, about 8-fold increase in apoptotic cell population was observed when Bcr-*abl* expression was specifically silenced by expression vector (Fig. 5B).

Bcr-*abl* silencing and proliferation rate of K562 cells. The role of Bcr-*abl* in hyper-proliferation of the leukemic blasts was also investigated. At each time point, cell growth was detected spectrophotometrically using the CellTiter AQ Proliferation Assay kit (Promega). The method is based on the detection of the number of viable cells in proliferation. In the presence of an electron coupling reagent (phenazine methosulfate), a tetrazolium compound (MTS) changed into a

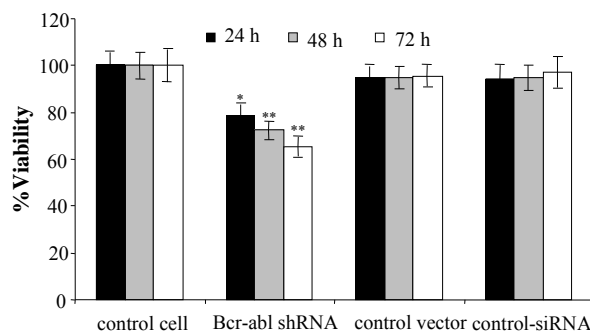


Fig. 3. Cell growth of k562 cells treated and untreated with specific anti-Bcr-*abl* siRNA expression vector. Viable cells were counted by trypan blue exclusion during suspension cultures after transfection with anti-Bcr-*abl* siRNA, siControl, vector control and GFP expression vector (n=3), * $P < 0.05$, ** $P < 0.01$.

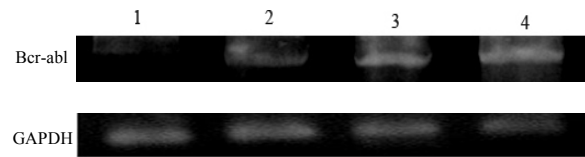


Fig. 4. Typical RT-PCR (48 h following transfection) showing the level of mRNA relative to that of Bcr-*abl* and GAPDH in k562 cells treated and untreated with anti-Bcr-*abl* siRNA (lane 1), siControl (lane 2), vector control (lane 3) and normal cell (lane 4).

formazan product. The absorbance of formazan product at 490 nm (OD_{490nm}) was recorded using 96-well plate reader Biotek (Power wave Xs2). Surprisingly, silencing of Bcr-*abl* using sib3a2-1 expression vector has a relatively long-term effect on cell proliferation capacity (data was not shown). These cells are unable to actively divide for at least 20 days after transient silencing of Bcr-*abl* in comparison to siControl transfected cells. To evaluate this effect, 3 days after the sib3a2-1 treatment, the cells were washed extensively to exclude dead cells or debris, recounted and replated for proliferation assay and similar results were observed.

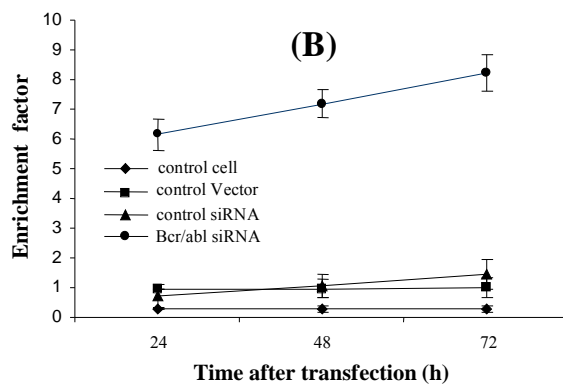
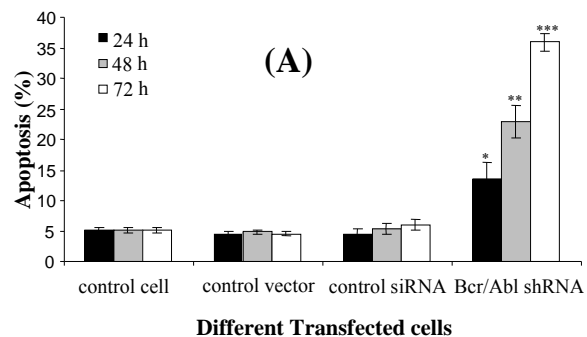


Fig. 5. (A) A time-dependently increase in the apoptotic cell population when treated with sib3a2 (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; (B) eight-fold increase in apoptotic cell population was observed in specifically silenced Bcr-*abl* cells using b3a2-1 siRNA.

DISCUSSION

In recent years, preliminary reports indicate that silencing of Bcr-abl using RNAi consistently reduces its expression. However, so far the molecular consequence of silencing of Bcr-abl has not been studied systematically. The previous studies showed that chemically synthesized siRNA specifically silenced the target gene [30-32]. This study was performed on Bcr-abl in K562 cell [33]. Chemically synthesized siRNA has a short half-life and therefore is needed a high and constant concentration of siRNA transfected to target cells.

The aim of this study was using siRNA expression vector as a suitable system for long term silencing of Bcr-abl in K562 cells. Data obtained show that the gene suppression is mediated by siRNA expression vector in K562 cells. Specifically, expressed siRNA induced a specific but transient reduction in Bcr-abl mRNA, and an inhibition of Bcr-abl-mediated cell proliferation as expected for mammalian cells [34].

The fact that a 72 h persistent reduced level of the Bcr-abl transcript was observed at 24 hours after transfection may be due to (a) the oligonucleotide degradation occurring under intracellular conditions and (b) a cell division was significantly reduced during the study. However, this phenomenon with synthetic siRNA was detected only 24 hours following electroporation [35]. This difference in response time is likely to reflect a long living of siRNA expression by vector and its proliferative effect on K562 cells. In fact, while the RNA induced silencing complex -mediated cleavage of mRNA promoted by synthetic siRNA is a transient process, the siRNA expression by pRNAH1.1/Neo vector is significantly longer. Alternatively, the inhibition of Bcr-abl expression by siRNA or antisense sequences [36, 37] compared with blocking Bcr-abl kinase activity by STI571 may induce different phenotypes in cytokine-supplemented cultures of Bcr-abl-positive cells [36]. The molecular basis for these differences and the kinetics of RNAi, triggered by exogenous or endogenous expression of siRNA [38], should be analyzed to find out the role of RNAi as a scientific tool or potential therapy in human hematopoietic cells. In this study, a unique sequence present in the Philadelphia chromosome of K562 cells has been targeted. The designed b3a2-1 showed a strong capacity to knockdown the transcript levels. The potency of expressed SiRNA using expression vector to silence the chimeric Bcr-abl gene is higher than that of synthetic siRNA, in terms of transcript, reduced proliferation, and activation of apoptosis

[38]. Bcr-abl has been reported to increase apoptosis resistance. Results of this study showed that treatment with vector-based on expression of specific siRNA against Bcr-abl leads to an increased apoptosis. K562 cell treatment with sib3a2-1 shows an increase in the apoptotic cell population. Nevertheless, about 8-fold increase in apoptotic cell population was observed when Bcr-abl expression was specifically silenced.

In transients silencing experiment using the vector based on expression of siRNA, 30% of Bcr-abl mRNA level is restored within 72 h. Hence, only a transient change in cell proliferation was expected. Surprisingly, in sib3a2-1-treated cell population, silencing of Bcr-abl has a relatively long-term effect on their proliferation capacity. These cells are unable to actively divide for at least 2 weeks after transient silencing of Bcr-abl in comparison to the siControl transfected cells. One could argue that this effect could be due to the presence of apoptotic cells or that the surviving resting cells recover slowly after 5-7 days of transient Bcr-abl silencing. However, following an extensive washing of the treated cells to exclude dead cells or debris and replated for proliferation assay, it was observed that transient Bcr-abl silencing has a profound proliferative defect.

In conclusion, the present study shows that siRNA expression vector is a powerful molecule to down-regulate *Bcr-abl* in Philadelphia-positive leukemic cell. When it is used, an enhanced anti-proliferative effect on K562 cells is observed. Although *in vitro* studies are not always predictive of clinical activities, results show that siRNA expression vector, may be used for treatment of leukemia and for the purging of Philadelphia-positive cells.

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