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Multiple gene silencing in *STAT* pathway in K562 cells

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ABSTRACT

Context: Chronic myeloid leukemia (CML) is characterized by the presence of a fusion oncoprotein *BCR-ABL*. This mutation imparts a constitutive phosphorylation activity of tyrosine residues in the cellular proteins. One of the targets of *BCR-ABL* is the *STAT5* protein, which when phosphorylated induces gene expression of antiapoptotic proteins such as *BCL-XL*. The *STAT* pathway has been targeted in the past by disrupting any one protein only. A multiple gene silencing has never been done in this pathway.

Aim: The aim of this study was to compare the effects of downregulation of *BCR-ABL*, *STAT5A*, *STAT5B,* and *BCL-XL*, individually and simultaneously, in human CML cell line (K562 cells) through RNA interference (RNAi). Further, gene expression, inhibition of proliferation, and apoptosis induction were assessed in K562 cells.

Materials and Methods: K562 cells were transfected with various combinations of small iRNA (siRNA) and the expressions of aforesaid genes were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. K562 cell proliferation and apoptosis were analyzed using 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and flow cytometry, respectively. The results were compared through one-way analysis of variance.

Results: qPCR and western blotting results post-siRNA transfection confirmed the targeted gene suppression and protein reduction in K562 cells. The cell proliferation assay and apoptosis assay revealed that simultaneous gene silencing of *BCR-ABL*, *STAT5A*, *STAT5B,* and *BCL-XL* had the highest killing effect on K562 cells as compared to knocking down these genes individually or in any other combinations.

Conclusions: This was the first time it was shown that multiple gene silencing in *STAT* pathway in CML cell line K562 was better as compared to individual gene silencing.

Keywords: *BCL-XL, BCR-ABL*, K562, Small interference RNA, *STAT5*

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INTRODUCTION

It is estimated that there will be 23.6 million new cases of cancer each year by 2030. Chronic myeloid leukemia (CML) itself has an incidence of 1–2 cases per 100,000 adults and it accounts for 30–60% of all adult leukemia in India.[1] CML is identified by a reciprocal translocation t(9;22) (q34;q11), known as Philadelphia (Ph) chromosome. This results in the formation of a unique gene product (P210) displaying a constitutive tyrosine kinase activity. The deregulated tyrosine kinase transforms healthy cells into cancerous and has become a primary target for the treatment of CML.[2] The Ph chromosome has been observed to be present in the bone marrow in 95% of cases of CML.[3]

The standard first line of treatment for patients in chronic phase of CML is tyrosine kinase inhibitor (TKI), imatinib mesylate (trade name: Gleevec). Imatinib mesylate selectively induces the growth arrest and the apoptosis of *BCR-ABL*-positive leukemia cells.[4] Though Imatinib was widely used for CML, a mutation known as T315I, where threonine was substituted with isoleucine, rendered the drug no longer effective

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to bind to the ATP domain in *BCR-ABL* oncoprotein.[5] Except for a third-generation TKI (ponatinib), all the other TKIs (dasatinib, nilotinib, and bosutinib) are not effective against T315I mutation in the kinase domain of *BCR-ABL* oncoprotein.[6,7] Moreover, mutations might occur in various genes of a pathway with variable frequencies. Along with targeting various key regulators in cancer, it would be a better approach to target an entire pathway in any cancer.[8]

BCR-ABL fusion gene plays the central role in CML and has been shown to participate in many pathways such as *MAPK, RAS, RAF, JUN* kinase, and *MYC*.[9] One of the important pathways in CML is the *STAT* pathway which includes *STAT5* gene as a downstream component of the *JAK/STAT* pathway. *BCR-ABL* directly instigates the tyrosine phosphorylation and dimerization of *STAT5*, followed by translocation of *STAT5* dimers to the nucleus, wherein they activate many antiapoptotic genes.[10] It has also been reported that *BCL-XL* is induced by *BCR-ABL* through the activation of *STAT5* in cell lines from CML patients.[11]

Small interference RNA (iRNA) therapy is speedily finding its way to get established as a dependable line of treatment.[12] Several researchers have tried to silence the CML-associated genes individually at the mRNA level such as *BCR-ABL,*[13] *STAT5,*[14] *BCL-XL,*[15] and many other genes.[16] Some researchers have tried silencing two genes in CML[17] and others have tried silencing multiple genes in various disorders such as breast cancer.[18]

Therefore, in this study, we hypothesized that the simultaneous gene silencing of the key oncogene (*BCR-ABL*) in CML and additional *STAT5* pathway disruption might be highly effective in killing K562 cells (human CML cell line). For this purpose, siRNA against *BCR-ABL*, *STAT5A, STAT5B,* and *BCL-XL* was transfected in K562 in all the 15 possible gene combinations. The effect of downregulating *BCR-ABL, STAT5A, STAT5B,* and *BCL-XL* gene expressions was analyzed by studying the proliferation inhibition and apoptosis induction in K562 cells. Comparing the results obtained, it was concluded that silencing all four genes together induced a higher rate of apoptosis in K562 cells.

MATERIALS AND METHODS

Cell Culturing

K562 cells were obtained from the National Center for Cell Science (Pune, India). The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics penicillin (100 kU/L) and streptomycin (100 mg/L) at 37°C in a humidified incubator with 5% CO_2.

Gene Knockdown in K562

ON-TARGETplus siRNAs for *BCR-ABL*, *STAT5A*, *STAT5B,* and *BCL-XL* were purchased from Dharmacon, USA. *BCR-ABL* siRNA was synthesized using the following sequence: 5′GCAGAGUUCAAAAGCCCUUdTdT 3,[13] and the remaining siRNAs were ordered off the shelf. From our previous experiment, it was decided to use 50 nM of each siRNA for 48 h for every experiment unless specified otherwise. However, to ensure comparability among experiments, a total amount of 200 nM siRNA were always used. For example, when only one target siRNA (50 nM) was used, then the remaining 150 nM concentration was filled by non-targeting (NT) siRNA to attain a total concentration of 200 nM. Similarly, when two siRNAs (50 nM + 50 nM) targeting two different genes were used, then 100 nM of NT siRNA was added to make the final concentration of 200 nM. Moreover, finally, when three siRNAs (150 nM) targeting three different genes were used, then 50 nM of NT siRNA was added to the mixture. No NT siRNA was added when all four siRNAs were used together as the total concentration of the siRNA mixture was already 200 nM. Although in the text, only the concentration of target siRNA used would be mentioned. The control cells were always transfected using 200 nM of NT siRNA for 48 h in all the experiments performed.

All of the transfections were conducted using a HiPerFect Transfection Kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, 2×10^5 K562 cells were plated per well of a 24-well plate in 100 µl serum containing IMDM. The required amount of siRNA was diluted in 100 µl serum-free IMDM, and 6 µl of HiPerFect Transfection Reagent was dissolved to the diluted siRNA for 5 min at room temperature. The complex (106 µl) was added to the cells, gently shaken, and thoroughly incorporated. After 6 h, 400 µl culture medium containing serum was added to the cells and incubated until further analysis. siRNA transfection efficiency was analyzed by counting positively transfected cells by SiGLO Red siRNA (Dharmacon, USA) per 100 cells under fluorescent microscope. The cells transfected with only Transfection Reagent HiPerFect were used as reference sample and cells transfected with NT siRNA (Dharmacon, USA) were used as control for all experiments.

Quantitative Polymerase Chain Reaction (Qpcr)

Total RNA was extracted from the sample by NucleoSpin® RNA/Protein kit by Macherey-Nagel GmbH & Co. (Germany) and reversed transcribed into cDNA by utilizing High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The primer sequences were as follows:

BCR-ABL forward, 5'- GTGTGAAACTCCAGACTGTC -3', and reverse, 5'- CAAAATCATACAGTGCAACGA -3',[19]

STAT5A forward, 5'- GAAGCTGAACGTGCAC-ATGAATC -3', and reverse, 5'- GTAGGGACAGAGTCTTCACCTGG -3',[14]

STAT5B forward, 5'- AGTTTGATTCTCAGGAAAGAATGT -3', and reverse, 5'- TCCATCAACA-GCTTTAGCAGT -3',[14]

BCL-XL forward, 5'- TGCATTGTTCCCATAGAGTTCCA -3', and reverse, 5'- CCTGAATGACCACCTAGAGCCTT -3',[20] and

GAPDH forward, 5'- GTCAACGGATTTGGTCGTATTG -3', and reverse, 5'- CATGGGTGGAATCATATTGGAA -3'.[21]

qPCR was performed in StepOnePlus™ real time-PCR System (Applied Biosystems, USA). Triplicate PCRs (20 µl) were performed using SYBR™ Select Master Mix (Applied Biosystems, USA). The reaction mixture was initially set at 50°C for 2 min for UDG activation and then at 95°C for 2 min for AmpliTaq® DNA

Polymerase, UP Activation and then subjected to 40 PCR cycles of 95°C for 3 s and 60°C for 30 s. A reference sample (K562 treated with Negative control [water]) was used to normalize data across experiments and mRNA levels were normalized to GAPDH levels.

Cell Proliferation Assay

Cell proliferation was determined using Cell Proliferation Kit I (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide [MTT]) by Roche, Basel, Switzerland. The K562 was seeded in 24-well plates at a density of 2×10^5 cells/well. Each well was transfected with a required amount of siRNAs. Cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO_2. After 48 h of incubation, 60 µl of MTT (0.5 mg/ml) was added in each well and incubated for another 4 h. To dissolve the precipitate, 600 µl of dimethyl sulfoxide was then added to each well. The proliferation of K562 cells was quantified at an optical density of 490 nM. Cell viability was calculated as the percentage of amount of treated cells on control cells (cells treated with NT siRNA).

Apoptosis Detection Assay

The cell apoptotic rate was evaluated using a Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen, USA). The K562 cells in the logarithmic phase were collected, centrifuged at 1000 rpm for 5 min, and washed with precooled phosphate-buffered saline. ×1 binding buffer was added to the cell pellet to achieve a concentration of 1×10^6 cells/ml. 5 µl of FITC Annexin V and 5 µl propidium iodide (PI) were added to this cell suspension and incubated at RT for 15 min. 400 µl of ×1 binding buffer was added to each tube and analyzed by flow cytometry within 1 h.

Western Blot Analysis

Total proteins were extracted from the cells using NucleoSpin® RNA/Protein Kit by Macherey-Nagel GmbH & Co. (Germany) following the manufacturer's protocol. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by loading 15 µg of protein in each lane. Proteins were then transferred to nitrocellulose membranes and then incubated with specific antibodies. The membranes were washed with Tris-buffered saline and Tween-20 and incubated with horseradish peroxidase (HRP)-conjugated second antibody for 1 h at room temperature (25°C). The blot was developed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Scientific, USA, and the signal was exposed with X-ray film. All antibodies were from Thermo Scientific, USA and they were used in the following dilutions - GAPDH (1:5000), *BCR-ABL* (1:500), *STAT5A* (1:1000), *STAT5B* (1:1000), *BCL-XL* (1:250), and Goat anti-mouse immunoglobulin G (H+L) secondary antibody HRP (1:50,000).

Statistical Analysis

Results were expressed as mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. GraphPad Prism 7 (GraphPad Software, Inc. CA, USA) was used for statistical analysis. The results were compared through one-way analysis of variance and differences between values were considered to be statistically significant at $P < 0.05$.

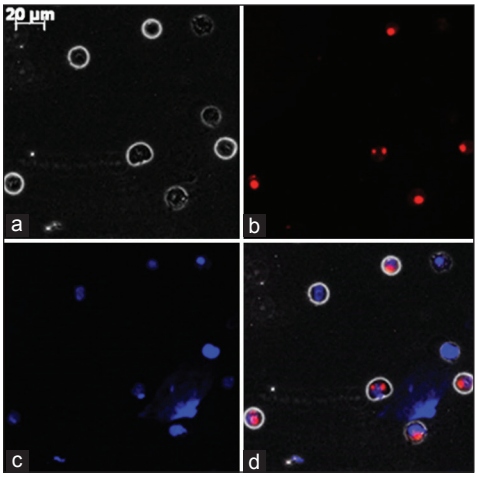

Figure 1: Fluorescent microscopy image of K562 cells: K562 cell was transfected with 50 nM small interference (si) GLO Red siRNA for 24 h and observed under fluorescent microscopy at ×400 magnification. (a) Phase contrast image of K562. (b) siGLO siRNA seen as red dots. (c) Nuclear region stained in blue by DAPI. (d) Merge of all the three images

RESULTS

Confirmation of siRNA Transfection

siGLO Red siRNA was used to confirm the successful transfection into K562 cells. K562 cell was transfected with 50 nmol/l (nM) siGLO Red siRNA for 24 h. Further analysis was performed using fluorescent microscopy. From Figure 1, it can be confirmed that the siRNA has entered the cells and got localized near the nuclear region. The transfection efficiency was determined to be 69 ± 6.5% when 50 nM siRNA was used for 24 h by counting positively transfected cells per 100 cells in the field of view. HiPerFect transfection technique showed 9 ± 3.5% of cytotoxicity when compared with non-transfected cells.

To determine whether 50 nM of each siRNA significantly reduced the gene expression in K562 cells, we transfected the cells with 50 nM of *BCR-ABL*, *STAT5A*, *STAT5B,* and *BCL-XL* siRNA individually for 48 h. After 48 h, the gene expression was detected through qPCR analysis and western blot analysis. The qPCR results showed that the mRNA level of each gene was significantly lowered in the transfected K562 cells than control cells (NT siRNA transfected K562 cells). The western blot results correlated with the qPCR results where the protein band intensity reduction was seen in siRNA-treated cells [Figure 2].

Effect of Silencing Single Gene

First, we wanted to study the effect of each siRNA on the gene expressions, cell proliferation, and apoptosis of K562. For this purpose, 50 nM of each siRNA was transfected into K562 individually, and gene expression of all four genes was studied

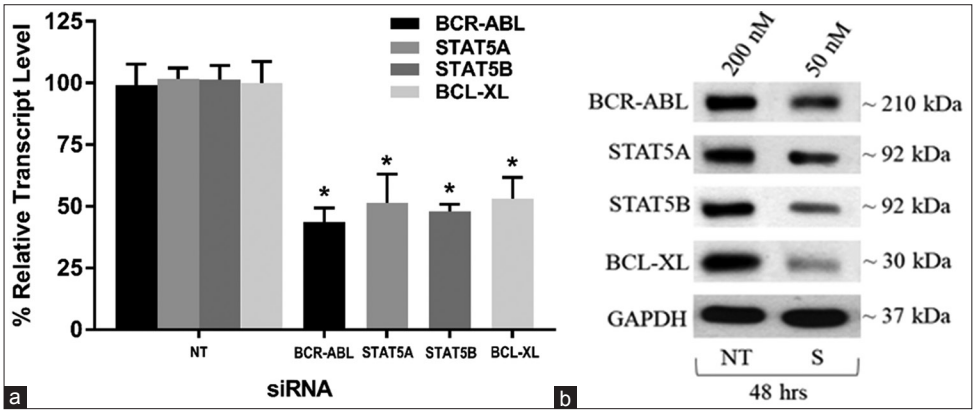

Figure 2: Confirmation of small interference (si) RNA transfection: K562 was transfected with 50 nM each of BCR-ABL, STAT5A, STAT5B, and BCL-XL siRNA individually for 48 h. (a) Gene expression: The relative transcript level of each gene is expressed in percentage as compared to gene expression in control. *GAPDH* gene expression was used to normalize the expression of other target genes. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when each value compared with their respective control. (b) Western Blot: With each sample, western blotting was carried out for the specific protein which was silenced with the respective siRNA. NT - K562 treated with 200 nM non-targeting siRNA, S - K562 cells treated with respective siRNAs at mentioned concentration and time point. The blot shown is a representative figure of three individual experiments

after 48 h. When *BCR-ABL* and *STAT5A* genes were silenced individually, the *BCL-XL* expression was lowered than the control sample by around 10%. Similarly, when *STAT5A* and *BCL-XL* gene was suppressed, the expression of *BCR-ABL* was lowered as compared to the control by approximately 10%. None of the above-mentioned difference was statistically significant, except when the target gene suppression was determined for each of the siRNA used [Figure 3a]. MTT test results revealed that the cell proliferation rate of the transfected cells was lower than that of the control cells. The lowest proliferation rate was observed when *BCL-XL* siRNA was used and it was found to be 66.34 ± 9.7% [Figure 3b].

In the previous experiments, all the four siRNAs could inhibit K562 cells proliferation to some extent. To investigate whether these silencing of genes accelerates K562 cells apoptosis, Annexin V/PI staining was carried out followed by flow cytometry analysis. Compared to the control cells, the apoptotic cells increased highest by 24 ± 13.7% in *BCL-XL* silenced cell population and lowest by 20 ± 9.1% in *BCR-ABL* silenced cell population. None of the results were statistically significant when compared with control cells. However, these results indicated that silencing *BCR-ABL, STAT5A, STAT5B, and BCL-XL* individually induced K562 cells to undergo apoptosis [Figure 4].

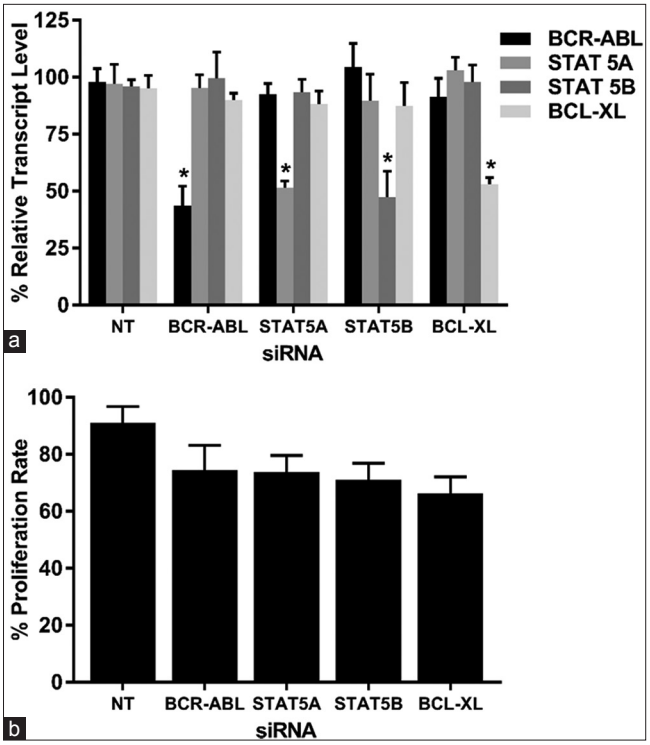

Figure 3: Effect of silencing single gene: K562 was transfected with 50 nM each of BCR-ABL, STAT5A, STAT5B, and BCL-XL siRNA individually for 48 h. (a) Gene expression: The relative transcript level of each gene is expressed in % as compared to gene expression in control. *GAPDH* gene expression was used to normalize the expression of other target genes. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when each value compared with their respective control. (b) Cell proliferation: K562 proliferation rate is expressed in % as compared to the proliferation rate in control. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control

Effect of Silencing Two Genes

Second, we intended to study the effect of silencing two genes at a time in *STAT* pathway in K562. Since we had four target genes, a total number of combinations comprising two different siRNAs were six. Each siRNA was used at 50 nM concentration and the cells were incubated for 48 h. Every siRNA significantly reduced its target gene's expression by more than 50% when compared with the NT siRNA-treated control. It was observed that, in some instances, simultaneous treatment with two siRNAs showed improved effects in silencing of its target genes. For example, the gene silencing improved when *BCR-ABL* and *STAT5A* were silenced together (40.95 ± 14.5% and 45.58 ± 11.01%) as compared to when they were silenced individually [43.63 ± 13.7% and 51.54 ± 5.4%,

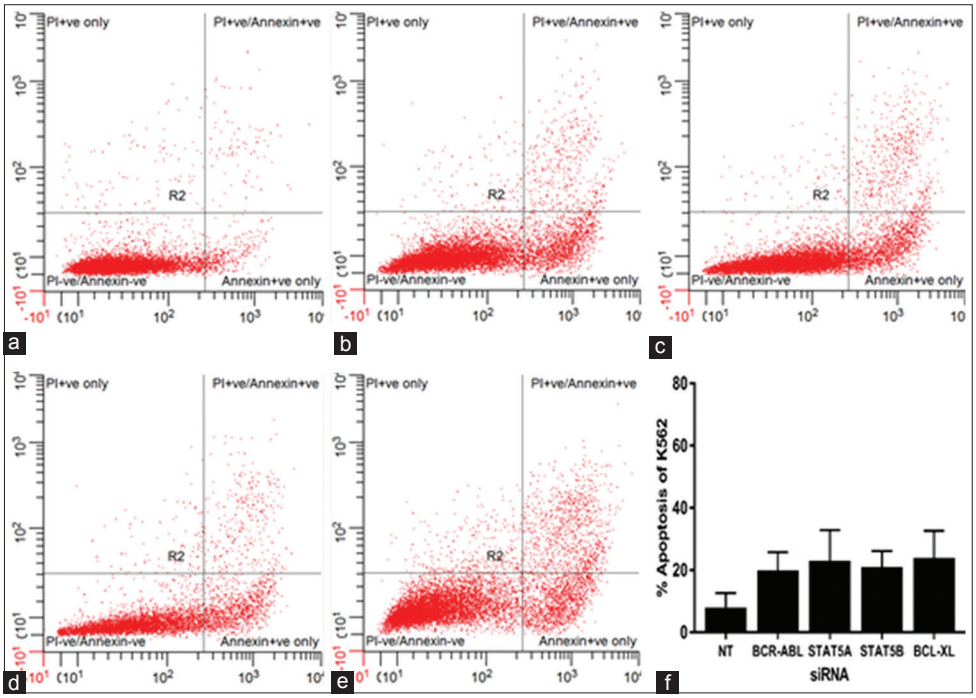

Figure 4: Apoptotic status after individual gene silencing: K562 cells were assayed through flow cytometry using Annexin V/PI staining. Data are representative of three separate independent experiments. (a) - With 200 nM of NT siRNA. (b) With 50 nM BCR-ABL small interference (si) RNA. (c) With 50 nM STAT5A siRNA. (d) With 50 nM STAT5B siRNA. (e) With 50 nM BCL-XL siRNA. (f) Apoptotic rate is expressed in % of Annexin V-positive events in a total number of events. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control

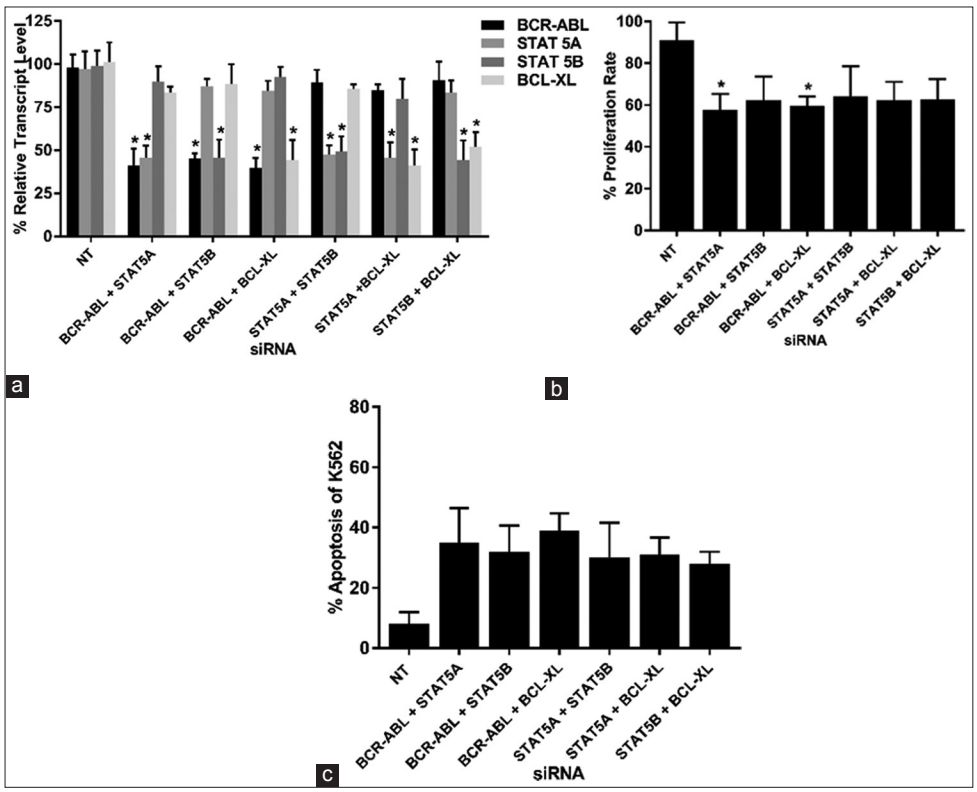

Figure 5: Effect of silencing two genes: K562 cells were transfected with different combinations of BCR-ABL, STAT5A, STAT5B, or BCL-XL siRNAs at 50 nM each for 48 h. (a) Gene expression: The relative transcript level of each gene is expressed in % as compared to gene expression in control. *GAPDH* gene expression was used to normalize the expression of other target genes. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when each value compared with their respective control. (b) Cell proliferation: K562 proliferation rate is expressed in % as compared to the proliferation rate in control. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control. (c) Apoptosis: K562 apoptotic rate is expressed in % of Annexin V-positive events in a total number of events. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control

Figure 3a]. Furthermore, *BCL-XL* showed improved silencing in all combination of siRNA [Figure 5a]. The cell proliferation rate was seen to be unambiguously reduced in all double siRNA combinations as compared to single siRNA application. Statistically significant reduction in cell proliferation was seen in two occasions: one when *BCR-ABL* and *STAT5A* siRNA were used together (57.8 ± 10.8%) and another when *BCR-ABL* and *BCL-XL* siRNA were used together (59.5 ± 8.2%) [Figure 5b]. More cells underwent apoptosis when two siRNAs were used together, and the highest apoptosis rate (39.74 ± 9.4%) was seen when *BCR-ABL* and *BCL-XL* siRNA were used together [Figure 5c].

Effect of Silencing Three Genes

The penultimate silencing strategy was to silence any three genes simultaneously and study its effect of gene silencing, cell proliferation, and apoptosis. In this category, four combinations of siRNA were possible with a total concentration of 150 nM for every combination. Every siRNA significantly reduced its target gene in all combination used. The non-targeted gene in every combination also showed reduced gene expression. The highest non-targeted reduction was seen in *BCL-XL* gene expression with 74.64 ± 13.6% when the other three genes were silenced [Figure 6a]. The cell proliferation in each combination

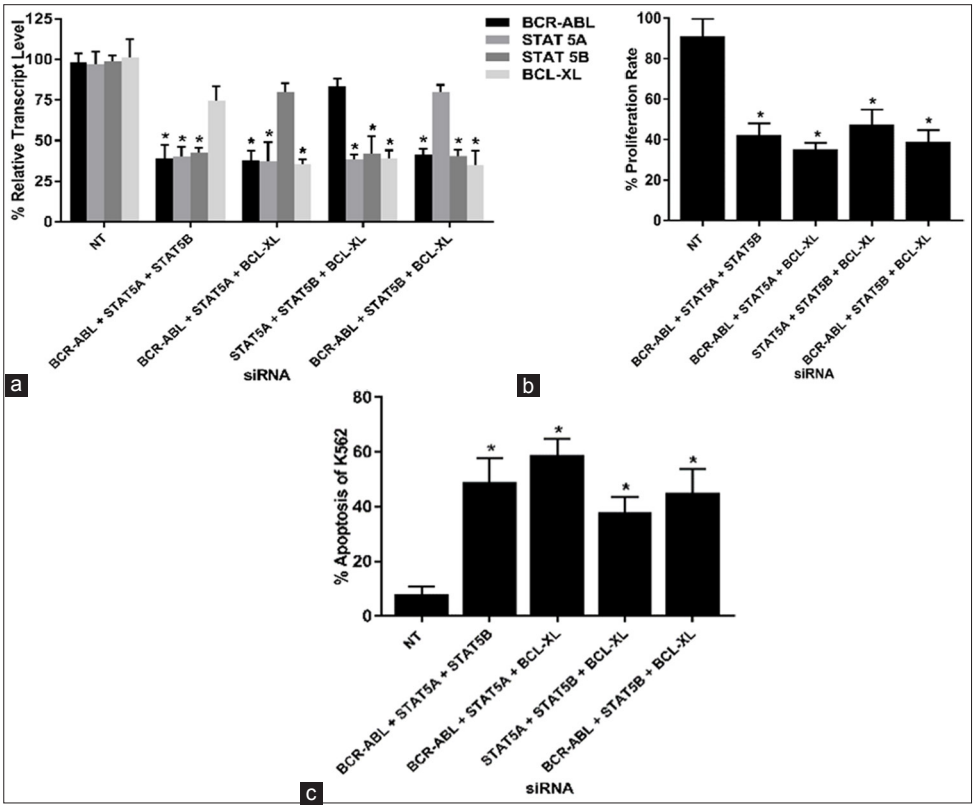

Figure 6: Effect of silencing three genes: K562 cells were transfected with different combinations of BCR-ABL, STAT5A, STAT5B, or BCL-XL siRNAs at 50 nM each for 48 h. (a) Gene expression: The relative transcript level of each gene is expressed in % as compared to gene expression in control. *GAPDH* gene expression was used to normalize the expression of other target genes. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when each value compared with their respective control. (b) Cell proliferation: K562 proliferation rate is expressed in % as compared to the proliferation rate in control. Values are expressed in % ± SD; $\boldsymbol{n} = 3$; $^*\boldsymbol{P} < 0.05$ when compared with control. (c) Apoptosis: K562 apoptotic rate is expressed in % of Annexin V-positive events in a total number of events. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control

Table 1: Effect of silencing all four genes in K562

siRNA used	A. % mRNA remaining				*B. Cell* proliferation rate (%)	*C. apoptosis* rate (%)
	BCR-ABL	STAT5A	STAT5B	BCL-XL		
NT	98.32±9.64	97.85±15.67	99.21±14.92	101.78±9.87	91.05±13.34	8.36±3.47
BCR-ABL+STAT5A+STAT5B+BCL-XL	33.01±10.2	36.94±9.81	40.77±12.93	31.17±11.63	25.21±9.25	64.15±10.47

K562 cells were transfected with BCR-ABL, STAT5A, STAT5B, and BCL-XL siRNAs together at 50 nM each for 48 h. A: Gene expression: The relative transcript level of each gene is expressed in %as compared to gene expression in control. *GAPDH* gene expression was used to normalize the expression of other target genes. Values are expressed in %±SD; n=3; Bold values - P<0.05 when each value compared with their respective control. B: Cell proliferation: Proliferation rate are expressed in % as compared to the proliferation rate in K562 cells treated with NT siRNA (control). Values are expressed in %±SD; n=3; Bold values P<0.05 when compared with Control. C: Apoptotic status: Apoptotic rate are expressed in % of Annexin V-positive events in a total number of events. Values are expressed in %±SD; n=3; Bold values P<0.05 when compared with control

was significantly reduced when compared with the NT siRNA-transfected control [Figure 6b]. The apoptotic rate of K562 increased significantly when three siRNAs were used together. In both the instances, the highest reduction in cell proliferation and an increase in apoptotic rate were seen when *BCR-ABL* siRNA was used along with *STAT5A* and *BCL-XL* siRNA [Figure 6c].

Effect of Silencing Four Genes

Finally, as a therapeutic approach, all four genes were silenced simultaneously with 50 nM concentration of each siRNA. The gene expression of all target genes was significantly reduced when compared with the control. When all four genes were silenced simultaneously, the gene expression of *BCL-XL* was reduced the most and *STAT5B* was reduced the least. The cell proliferation rate of K562 was reduced by around 75% and the apoptosis was induced in more than 60% of K562 cells when all four siRNAs were used together [Table 1].

Multiple Gene Silencing is Better to Individual Gene Silencing

It was evident from all the previous experiments that silencing *BCR-ABL*, *STAT5A*, *STAT5B,* and *BCL-XL* simultaneously was the most efficient way to kill K562 cells in this setup. We wanted to confirm that this was an additive effect of siRNAs and not merely a cause of higher concentration of siRNAs. To prove this, we individually transfected high concentration (200 nM) of each siRNA in K562 cells and compared the results with all 4 siRNA-transfected cells. Each siRNA at 200 nM concentration reduced its target gene to a range of 9–14%, while in all 4 siRNAs, silencing the genes was reduced to a range of 31–41% [Figure 7a]. High concentration of individual siRNA was not able to reduce cell proliferation to >41.5%, but all four siRNAs were able to reduce the cell proliferation rate to 25.21 ± 9.2% [Figure 7b]. Similarly, the highest rate of apoptosis (51.25 ± 14.97%) was induced when BCL-XL siRNA was used at 200 nM, but all four siRNAs were able to induce 64.15 ± 10.4% apoptosis in K562 cells [Figure 7c].

DISCUSSION

In CML, *BCR-ABL* displays constitutive phosphorylation activity of tyrosine residues in various cellular proteins.[22] Although *STAT5A* and *STAT5B* share a very similar structure, they have different functions in CML. *STAT5* and *AKT* together drive the oncogenesis in CML.[23] *BCL-XL* plays a very important role in cell survival and inhibits apoptosis in K562 cells.[15] This study investigated the effect of silencing *BCR-ABL, STAT5A, STAT5B,* and *BCL-XL* individually and simultaneously in different combinations. Gene expression profile, cell proliferation rate, and apoptosis induction rate were studied after transfection with siRNA/s. We could demonstrate that multiple siRNA treatments for K562 cells were highly effective as compared to single siRNA treatment for 48 h with 50 nM concentration of each siRNA.

Individually, 50 nM concentration of siRNA decreased mRNA, reduced cell proliferation, and increased apoptosis in K562 cells [Figures 3 and 4]. While single target inhibition showed only slight effects, the combined silencing caused a statistically significant decrease in cell proliferation and significantly increased the apoptosis rate in K562 cells [Table 1]. Simultaneous gene silencing was also the most efficient combination to inhibit cell proliferation and induce apoptosis compared to all other

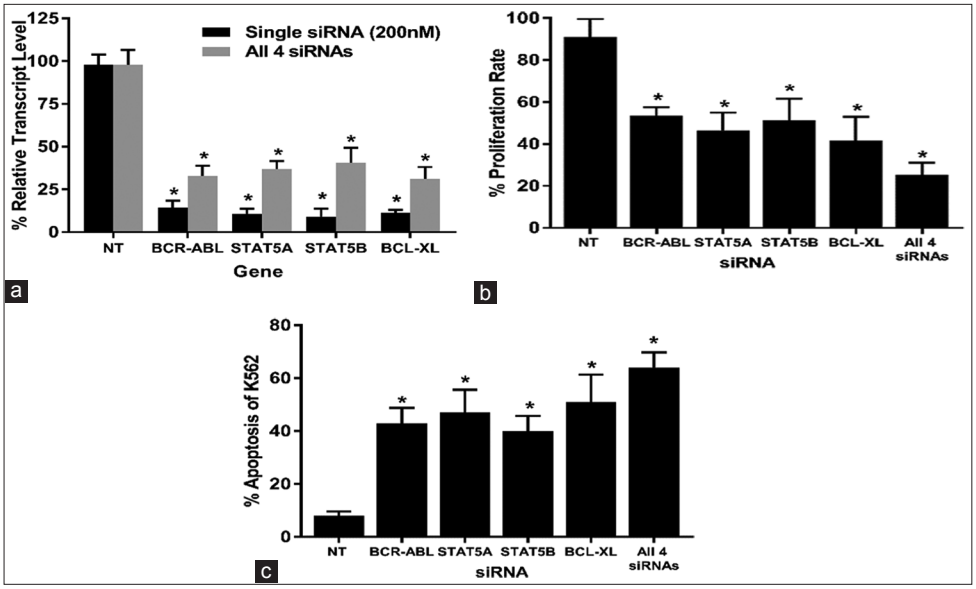

Figure 7: Comparison of individual silencing with high concentration small interference (siRNA) and simultaneous gene silencing. K562 cells were transfected individually with 200 nM of BCR-ABL, STAT5A, STAT5B, and BCL-XL siRNA and all 4 siRNAs with 50 nM of each siRNA together for 48 h. (a) Gene expression: The relative transcript level of each gene is expressed in % as compared to gene expression in Control. *GAPDH* gene expression was used to normalize the expression of other target genes. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when each value compared with their respective control. (b) Cell proliferation: K562 proliferation rate is expressed in % as compared to the proliferation rate in control. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control. (c) Apoptosis: K562 apoptotic rate is expressed in % of Annexin V-positive events in a total number of events. Values are expressed in % ± SD; $n = 3$; $^*P < 0.05$ when compared with control

combinations of siRNAs [Figures 5 and 6]. We were also able to demonstrate the non-target silencing effect where the *BCL-XL* expression was reduced when *STAT5A* and *STAT5B* were silenced together [Figure 5] similar to a work by Yanli *et al.*[24]

Targeting four genes in the *STAT* pathway was more effective in triggering apoptosis and inhibition of cell proliferation when compared to the silencing of three or less number of genes. This could be because, when a single gene is blocked, the cell switches to alternate pathways for survival. However, when a more number of pathway-related genes are silenced simultaneously, it becomes difficult for the cells to substitute for each blocked protein. Hence, simultaneous gene silencing blocks signaling pathways with or without additional targeting of the key regulatory gene/s. The basic toxicity of siRNAs is well studied and documented. Lowering the concentration of each siRNA would reduce the off-target effects and also avoids the RNAi endogenous pathway to get saturated with a single type of siRNA in a cell.[25] We were able to show that using all 4 siRNAs at 50 nM concentration each was much more effective than using any single siRNA at 200 nM concentration [Figure 7].

Certain shortcomings must be highlighted in the reported experiments. K562 cell line was the only cell model used in this study; we restricted the studies to this model due to its close comparison with CML disorder. Another limitation might be that the present study focused on only one of the many pathways in CML. Simultaneous gene silencing targeting key players in other pathways of CML would be an interesting approach in this kind of the study. Finally, *in vivo* experiment would have been helpful in ascertaining our findings in K562.

CONCLUSIONS

We were able to show that, in K562 cells, simultaneous silencing of *BCR-ABL, STAT5A, STAT5B,* and *BCL-XL* was more efficient in inhibiting cell proliferation and inducing apoptosis than silencing these genes individually or in any other combinations.

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