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Original Article

c-Myc Inhibition Induces an Additive Effect with Cyclophosphamide in Acute Lymphoblastic Leukemia

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Abstract

***Background*:** Cellular-myelocytomatosis (c-Myc), an oncoprotein and a transcription factor, is involved in several essential cellular processes. The c-Myc expression level is highly regulated in normal cells. It has been proved that c-Myc expression is deregulated in malignant cells due to rearrangements and mutations. The overexpression of this molecule is also reported to be present in acute lymphoblastic leukemia (ALL) as well, which is correlated with an unfavorable response to treatment, poor prognosis, and decreased overall survival. The upregulation of c-Myc results in increased proliferation, cell growth, and survival of ALL cells. Hence, making it an ideal target for leukemia treatment. This study evaluates the effect of c-Myc silencing combined with cyclophosphamide treatment, an FDA-approved chemotherapeutic.

Methods: Peripheral blood and bone marrow samples (mononuclear cells) were derived from eleven ALL patients. To silence c-Myc, small interfering-RNA (siRNA)-lipofectamine was used. The efficacy of gene silencing was assessed by the qRT-PCR test. Next, the effect of c-Myc silencing combined with cyclophosphamide treatment in ALL primary cells was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test.

Results: ALL cells were successfully transfected with c-Myc-siRNA. Also, treating cells with cyclophosphamide exerted a slight fall in the c-Myc mRNA level. The MTT test revealed that following the inhibition of c-Myc by siRNA, the viability of primary ALL cells decreased in response to cyclophosphamide treatment. Also, it was discovered that silencing c-Myc with siRNA combined with cyclophosphamide treatment significantly inhibits the growth of primary ALL cells compared to cyclophosphamide monotherapy.

Conclusion: c-Myc possesses high potential in the treatment of several cancers. Our findings add ALL to this category as well. Silencing c-Myc sensitizes ALL cells to cyclophosphamide treatment and can help with the better treatment of the afflicted individuals.

Introduction

Acute lymphoblastic leukemia (ALL), a very common childhood malignancy,[1] results from the uncontrolled proliferation of lymphoid progenitors.[2-4] Eighty-five percent of ALL cases account for B-cell lineage, while the rest affect T-cell progenitors.[5-7] Several mutations are reported to be involved in the process of ALL development.[8] ALL peaks at ages around 5 and 50.[9] Pediatric therapeutic regimens include corticosteroids, asparaginase, alkaloids, and antimetabolites.[10] Despite all the advances in chemotherapy strategies and recent improvements in response to treatments, only 30%-40% of adult ALL patients obtain long-term remission.[11] Therefore, new therapeutic approaches are required for the successful and effective treatment of ALL.

Cyclophosphamide, an alkylating agent, widely used to treat different neoplasms,[12] has been reported to possess immunosuppressive properties as well. Cyclophosphamide treatment combined with other chemotherapeutics to treat ALL is under study by several clinical studies. The agent is highly cytotoxic, therefore, immunotherapeutics-combined (which possess high specificity and less toxicity) cyclophosphamide treatment can reduce its required and effective dose [13]. Nowadays, small interfering RNA (siRNA) is widely used in cancer research to evaluate the role of different genes in the process of cancer.[14] SiRNAs induce the degradation of the target mRNA, therefore silencing the target gene.

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However, to safely deliver siRNAs into the cell, a vector/carrier is required.[14,15]

The *MYC* family genes comprise three distinct genes including *MYC (c-MYC), MYCN,* and *MYCL*. All three genes code for transcription factors with a helix-loop-helix leucine zipper structure.[16] Cellular-myelocytomatosis (c-Myc), a crucial cell cycle regulator, is coded by the *c-Myc* gene, located on 8q24.[17] c-Myc forms a heterodimer with Myc-Associated factor X (MAX) protein. The dimer is responsible for most of the c-Myc functions. C-Myc functions as a transcription factor downstream of several cellular signaling pathways, and therefore its expression level is highly regulated.[18] C-Myc highly regulates cell cycle progression, cell death, nucleotide metabolism, and proliferation.[19,20]

The expression of c-Myc is reported to be dysregulated in various malignancies.[20] Several hematological neoplasms have also been reported to show c-Myc aberrant expression, including Burkitt lymphoma (BL), diffuse large B cell lymphoma (DLBCL),[21,22] and multiple myeloma (MM).[23] Allen et al reported the c-Myc overexpression in B-ALL cells, which was correlated with poor prognosis, organomegaly, and elevated risk of long-term disease.[19] Based on another study, B-ALL cells express c-Myc, which correlates with the expression of P53, mutations of *TP53,* and decreased overall survival.[24] According to a case report, c-Myc rearrangement with Bcl-2 in a B-ALL patient severely weakened the patient's prognosis. The patient presented with a significant increase in extra-nodal infiltration and disease progression, which ended up in no response to treatment and death.[25]

In the case of T-ALL patients, adults, in particular, the cure rate is only around 50% and only 7% of the afflicted people maintain overall survival after five years.[26,27] Abnormal c-Myc expression keeps T-ALL leukemic stem cells active, and c-Myc silencing effectively stops leukemogenesis.[28] C-Myc has been reported to be essential for T-ALL development as well.[29,30] In addition, it has been found that through aberrant signaling of the NOTCH pathway, chromosomal translocations, and unknown mechanisms, c-Myc is upregulated and over-activated in T-ALL cells.[31]

According to a study, following the silencing of c-Myc, the viability of DLBCL cells in response to cyclophosphamide was decreased significantly, compared to cells only treated with cyclophosphamide [32]. However, the results in the case of ALL are scarce. Therefore, our study is one of the leading and most novel studies in this field.

Considering the sufficient evidence on the role of c-Myc in ALL and its progression, as well as the effect of silencing it in other hematological malignancies, and due to the scarcity of data regarding ALL, this study aimed to investigate the effect of silencing c-Myc in combination with cyclophosphamide treatment. To our knowledge, this is one of the most pioneering studies evaluating the effect of this combination therapy on these cells.

Methods

Materials

Cyclophosphamide was supplied by Cayman Chemical Company. Human c-Myc gene targeting siRNA was bought from Santa Cruz Biotechnology, Inc. (catalog number: sc-29226). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide MTT Assay Kit was supplied by the American Type Culture Collection (ATCC® 30-1010K).

Patient samples

Cellular samples were collected from eleven confirmed ALL patients at Shahid Ghazi Hospital, Tabriz, in accordance to the declaration of Helsinki. Patients' demographic data are presented in Table 1.[15] Using Ficoll Paque™ Plus (GE Healthcare, Uppsala, Sweden) and a centrifugation system, mononuclear cells were separated from the whole blood according to the instructions provided by the manufacturer. Next, the separated cells

Table 1. Patients' characteristics

Patients	Age (y)	Sex	WBC (x10^3/mL)	Platelet (x10^3/mL)	Hb (g/dL)	LDH level (Nl: Up to 480 U/L)	Hepatomegaly	Splenomegaly	Lymphadenopathy	C- ALL Ag (CD10) (%)	Subtype
1	3	F	106.3	23	8.2	1801	Yes	Yes	Peripheral	83	Pre B-cell
2	11	M	5.1	39	12.2	1372	Yes	Yes	No	90	Pre B-cell
3	4	M	2.5	52	6.6	395	Yes	Yes	Peripheral	23	Pre B-cell
4	10	M	8	141	10.3	360	Yes	Yes	Peripheral	0	Pre B-cell
5	8	M	10.4	66	8.7	701	No	Yes	No	92	Pre B-cell
6	8	M	4,02	74	9.8	1283	Yes	Yes	No	0	Pre B-cell
7	6	M	9.9	8	2.4	375	NO	Yes	No	79	Pre B-cell
8	11	M	6.5	54	9.8	2437	Yes	No	Peripheral	0	Pre B-cell
9	12	M	74.2	21	9.9	1284	Yes	Yes	Mediastinal	0	T-cell
10	4	M	41.3	53	11.5	1525	Yes	Yes	Mediastinal	0	T-cell
11	9	M	56.4	57	10.1	1367	Yes	Yes	Mediastinal	0	T-cell

Abbreviation: WBC, white blood cells; LDH, lactate dehydrogenase; CALL Ag, Common ALL antigen; Hb, hemoglobin.

were cultured in RPMI-1640 containing 20% FBS and 2% L-glutamine. Viable cells were counted prior to any downstream analysis.

Cell transfection with siRNA

The cells were seeded at a 1×10^5 concentration per well in 96 well plates and were incubated for 24 hours at 37 °C. Next, lipofectamine 2000 (Invitrogen) was used to transfect cells with siRNA. C-Myc siRNA, control siRNA, and lipofectamine were diluted at recommended concentrations based on the instructions of the manufacturer with the opti-MEM medium. Subsequently, the compounds were mixed and incubated for 20 minutes at 24 °C. Finally, the cells were treated with the compounds containing 50 pM of siRNA for 24 or 48 hours.

Gene expression analysis

After the cell transfection with siRNA, a qRT-PCR test was run to ensure the efficient transfection of cells. First, using TRIzol Reagent (Invitrogen) the total RNA of ALL cells extracted. RNA was then transcribed into cDNA and stored at -20 ℃. Next, SYBER Green PCR Master Mix (Thermo Fisher, US) was used to run a qRT-PCR with 1 µl of cDNA by a light-cycler 480 qRT-PCR system (Roche). The $2^{-\Delta\Delta CT}$ method was used to relatively measure the expression level of genes to β-actin mRNA level. The following primers were used: c-Myc Forward (F) primer: 5'-CCTGGTGCTCCATGAGGAGAC-3', c-Myc Reverse (R) primer: 5'- CAGACTCTGACCTTTTGCCAGG -3',[33] β-actin F primer: 5'- CACCATTGGCAATGAGCGGTTC -3', and β-actin R primer: 5'- AGGTCTTTGCGGATG TCCACGT -3'.[34]

Analysis of growth inhibition

To determine the Ic_{50} values for PBMCs and BMMCs, cells were cultured with increasing concentrations of the cyclophosphamide (5, 10, 15, 20, 25 µM). Subsequently, an MTT test was performed. The concentration that stopped cellular growth to 50% was calculated using GraphPad Prism V9.

To assess the effect of the combinational groups on the growth of cells, an MTT test was performed. Briefly, patient-derived cells were inoculated into a 96-well plate (1×10^4 cells/well) and incubated for 24 hours. Subsequently, cells were subjected to the following mixtures for 24 or 48 hours; untreated, lipofectamine, scramble siRNA, c-Myc siRNA, cyclophosphamide, lipofectamine-c-Myc siRNA, lipofectamine-c-Myc siRNA + cyclophosphamide, and DMSO (0.2%). 50 pM of siRNA and Ic_{50} of the drug were used in each group. Next, 10 µL of MTT reagent was added to each well and incubated for 4 hours. After incubating each well for 4 hours with 10 µL of MTT reagent, the medium was removed, and PBS was used to wash each well. A microplate reader (Thermo Fisher, Waltham, MA, USA) measured the absorbance of wells after 100 µL of DMSO was added to each well. Next, cell viability was calculated using this equation[35]:

$$\text{Viability} = \frac{\left(\text{OD treated well}\left[-\text{blank}\right]\right)}{\left(\text{mean OD control well}\left[-\text{blank}\right]\right)} \times 100$$

Statistical analysis

The data were statistically analyzed by GraphPad Prism V9 and SPSS. Statistical significance was set at $P < 0.05$.

Results

Cells were efficiency transfected with siRNA using lipofectamine

To make sure that cells were efficiently transfected with siRNA, a qRT-PCR test was run.

We evaluated the effect of our treatments on c-Myc mRNA levels. Treating cells with lipofectamine-c-Myc siRNA significantly decreased the mRNA level of c-Myc, indicating the effective transfection of lipofectamine. The result was significant compared to the results of cells treated with lipofectamine alone, control siRNA, c-Myc siRNA without a vector, and untreated.

Also, we discovered that the c-Myc mRNA level was slightly decreased when cells were subjected to cyclophosphamide. The findings of the qRT-PCR test are presented in Figure 1.

The viability of cells in response to cyclophosphamide was decreased following the silencing of c-Myc

The $IC_{50} = 22$ µM for BMMCs and $IC_{50} = 19$ µM for PBMCs were measured by MTT tests following 24 hours incubation of cells with cyclophosphamide (Supplementary file 1, Figure S1).

Next, we aimed to evaluate the co-treatment effect of c-Myc silencing with cyclophosphamide on the growth of cells by running an MTT test after 24 or 48 hours incubation with the various treatments. The results are presented in Figures 2a and 2b.

We noticed that treating cells with lipofectamine-c-Myc siRNA, resulted in a considerable fall in viability, even though this effect was not as considerable as the effect of cyclophosphamide treatment. Next, the combined treatment effect was evaluated. The results indicated that cells with silenced c-Myc and treated with cyclophosphamide demonstrated the highest level of apoptosis compared to treatment with cyclophosphamide alone. The results were comparable to the controls, including untreated, treated cells with lipofectamine, and bare siRNA.

Therefore, inhibition of c-Myc sensitizes ALL primary cells to cyclophosphamide and helps with the better elimination of the neoplastic cells. Also, we found the treatment effect to be increased over the subsequent 24 hours of incubation.

Discussion

ALL, a neoplastic disease of the hematopoietic system, is characterized by the repletion of lymphoblasts in several organs.[2,3] ALL in the elderly is associated with

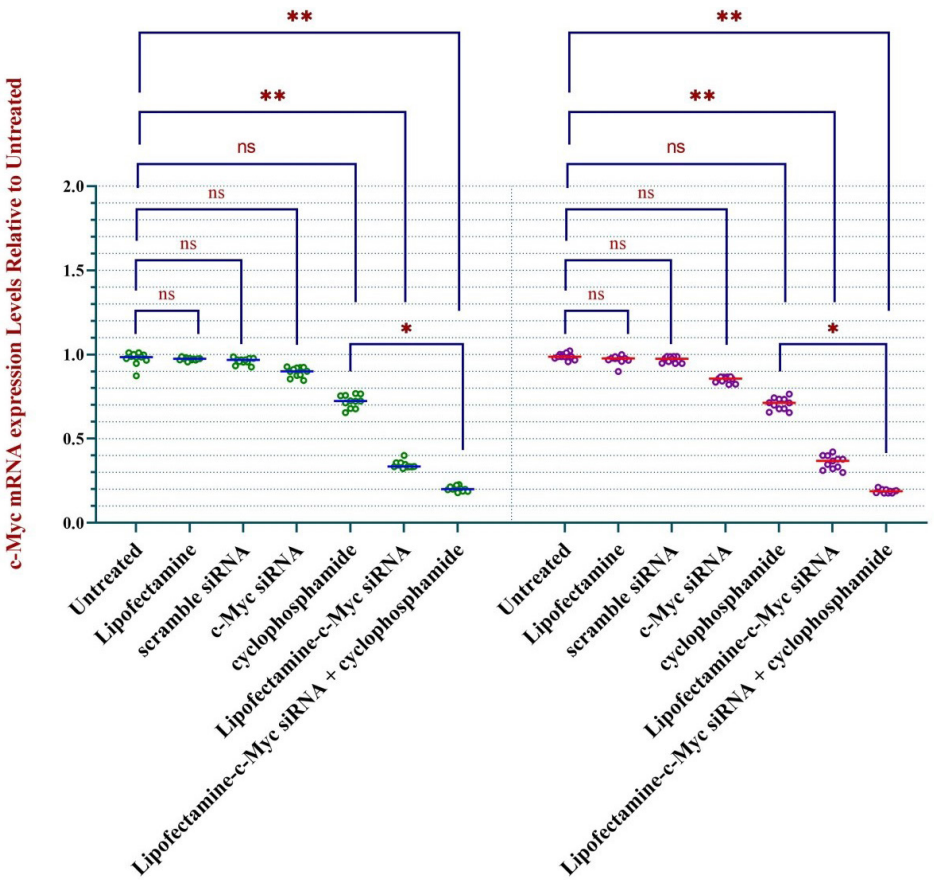

Figure 1. Treating ALL cells with anti-c-Myc siRNA silences the c-Myc gene. Transfecting mononuclear cells separated from peripheral blood (PB) and bone marrow (BM) of ALL patients (n = 11) with siRNA using lipofectamine silenced c-Myc gene, as investigated using qRT-PCR. * represents $p < 0.05$ and ** indicates $P < 0.01$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, bone marrow mononuclear cell; ns, non-significant

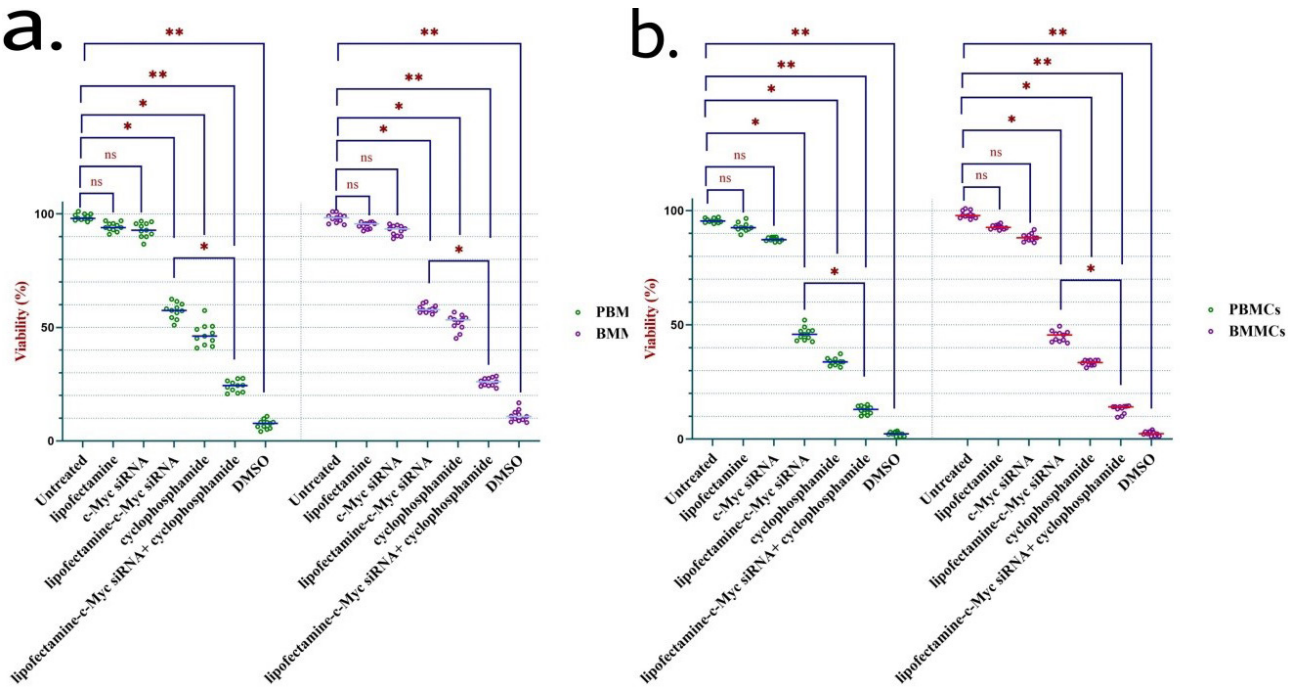

Figure 2. c-Myc silencing decreased the viability of ALL cells to cyclophosphamide treatment. Silencing *c-Myc* in mononuclear cells separated from peripheral blood (PB) and bone marrow (BM) of ALL patients (n = 11) enhanced the cytotoxicity of cyclophosphamide in ALL cells following 24 hours (2a) and 48 hours (2b) incubation, determined by MTT test. * represents $P < 0.05$ and ** indicates $P < 0.01$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, bone marrow mononuclear cell; ns, non-significant; DMSO, dimethyl sulfoxide

chemoresistance and therefore poor prognosis.[11] Thus, new approaches to cancer therapy are required for the successful treatment of ALL. Interestingly, c-Myc is reported to be highly involved in cancer course.

C-Myc, an oncoprotein, and a transcriptional factor dimerizes with MAX to bind the DNA molecule and regulate gene expression.[36] C-Myc is reported to induce cell growth, nucleotide and lipid synthesis, glucose metabolism, ribosome biogenesis, transcription, and translation of several genes.[37] Since c-Myc is considered an oncogene, it is strictly regulated in healthy cells.[38] Translocations, point mutations, and gene amplifications result in c-Myc deregulated expression which is reported in several types of neoplasms, hematopoietic malignancies in particular.[18,39] In BL, t (8;14)(q24;q32) induces high levels of c-Myc which is involved in the cancer course.[18,22] Many studies have suggested that c-Myc is upregulated in several cancers. T-ALL cells express high levels of c-Myc. It is proven that c-Myc expression is essential for the growth, and proliferation of T-ALL cells and the development of T-ALL.[40] The overexpression of c-Myc has been proven in B-ALL cells as well. This upregulation is directly correlated with the mutations of the *TP53* gene, unfavorable prognosis, and decreased overall survival of B-ALL patients.[19,24,25] Therefore, we aimed to silence this gene with siRNA and study its impact on the viability of cells in response to an FDA-approved anticancer drug, cyclophosphamide.

To efficiently transfect cells with siRNA, lipofectamine was used. As seen in Figure 1, lipofectamine-c-Myc siRNA treatment significantly decreased the mRNA level of c-Myc. In addition, the c-Myc mRNA level was found to be slightly decreased in response to cyclophosphamide treatment. In the cell group receiving both siRNA and cyclophosphamide, the lowest level of c-Myc mRNA was noted. This was evident in both BMMCs and PBMCs. Next, the result of gene silencing on the growth of cells was evaluated by an MTT test. We found that silencing c-Myc sensitizes both T-ALL and B-ALL cells to cyclophosphamide treatment, as the rate of growth inhibition was considerably elevated in this group compared to cyclophosphamide monotherapy. Moreover, the effect of the treatments was further augmented over the next 24 hours incubation. Therefore, c-Myc inhibition possesses high potential in eliminating ALL cells.

Our findings are in support of several studies evaluating the role of c-Myc in cancer. Akyurek et al report the correlation between c-Myc rearrangements, decreased overall survival, and poor prognosis in DLBCL patients.[41] According to Kendrick et al study, silencing c-Myc sensitizes DLBCL cells to cyclophosphamide treatment. Also, the combinational therapy induces a higher level of caspase-3 activity compared to cyclophosphamide monotherapy.[32] Moreover, Skorski et al reported that following the inhibition of *c-Myc*, the proliferation of chronic myeloid leukemia (CML) cells was significantly decreased, which was comparable to the silencing of the *BCR-ABL* gene. The same research reports finding a synergism between *BCR-ABL* and *c-Myc* silencing. They added that the survival of CML mice xenograft models was improved following the silencing of the *c-Myc* gene.[42,43] Therefore, not only c-Myc expression possess prognostic value in cancers but also synergizes with several anti-cancer drugs in eliminating neoplastic cells.

In this study, no significant difference was observed between patients' samples in response to the treatments, which could be due to the scarcity of sample numbers. Therefore, future studies can examine the effect of this treatment in different subgroups of ALL as well as different samples of patients with larger statistical populations. In addition, the effect of this treatment can be studied with more advanced methods in leukemic stem cells to eliminate minimal residual disease, which was beyond the scope of this study. Also, studies can be conducted in vivo to measure survival following treatment.

Conclusion

To conclude, silencing c-Myc with siRNA decreases the viability of ALL cells. In addition, silencing c-Myc significantly sensitizes ALL cells to cyclophosphamide treatment. Future studies can confirm the existence of this additive effect between these two treatments with more samples and also investigate the effect of this combined treatment on different subtypes of ALL. Additionally, this data can help provide a new therapy approach for ALL patients with poor prognosis, due to its potential in improved elimination of ALL cells.

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Authors' Contribution

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Methodology: Mohammad Sadeghi.
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Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Ethical Approval

This study was approved by the ethics review board of Tabriz University of Medical Sciences (the ethical code: IR.TBZMED.REC.1399.256).

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Supplementary File
Supplementary file contains Figure S1.

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