Knockout of BIRC5 Gene by CRISPR/Cas9 Induces Apoptosis and Inhibits Cell Proliferation in Leukemic Cell Lines, HL60 and KG1

Introduction: Human Baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) which encodes survivin exhibits multiple biological activities, such as cell proliferation and apoptosis. Survivin is overexpressed in numerous malignant diseases including acute myeloid leukemia (AML). Recent studies have shown that the CRISPR/Cas9 nuclease-mediated gene-editing systems are suitable approaches for editing or knocking out various genes including oncogenes.

Methods and materials: We used CRISPR-Cas9 to knockout the BIRC5 in the human leukemic cell line, HL60, and KG1, and these cell lines were transfected with either the Cas9- and three sgRNAs expressing plasmids or negative control (scramble) using Lipofectamine 3000. The efficacy of the transfection was determined by quantitative reverse transcription-polymerase chain (RT-qPCR) and surveyor mutation assays. Cell proliferation and apoptosis were measured by MTT assay and flow cytometry, respectively.

Results: We have successfully knocked out the BIRC5 gene in these leukemic cells and observed that the BIRC5-knocked out cells by CRISPR/Cas9 showed a significant decrease (30 folds) of survivin at mRNA levels. Moreover, cell death and apoptosis were significantly induced in BIRC5-CRISPR/Cas9-transfected cells compared to the scramble vector.

Conclusion: We demonstrated for the first time that targeting BIRC5 by CRISPR/Cas9 technology is a suitable approach for the induction of apoptosis in leukemic cells. However, further studies targeting this gene in primary leukemic cells are required.

Keywords: BIRC5, survivin, CRISPR/Cas9 nuclease, AML, KG1 cells, HL60 cell
Human Baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), belongs to the inhibitor of apoptosis (IAP) family, encodes survivin protein as a bifunctional regulator of apoptosis inhibition and cell cycle progression. An increasing number of studies have revealed that survivin preferentially upregulated in leukemia and fetal cells, but it does not exist in normal differentiated adult tissues.\(^9\)\(^\text{-}12\) Since a strong correlation between the elevated survivin expressions and clinicopathological features of a number of cancers has been reported, many Researchers paid attention to target survivin by employing various kinds of molecular and cellular technologies such as antisense oligonucleotides targeting survivin mRNA,\(^13\) induction of specific cytotoxic T cells and generation of antibody against it,\(^14\) however, most of these preclinical studies were not clinically successful.

Through technological breakthroughs in the field of biotechnology and molecular medicine over the recent years, now we have come in the “gene editing era”. High-throughput genome engineering tools would break new ground by preventing and/or treating the most devastating genetic disorders. In the last decades or so, targetted-specific nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the “clustered regularly interspaced short palindromic repeats” (CRISPR)/“CRISPR-associated genes 9” (Cas9), have extremely been used in genetic modification industry.\(^15\)\(^\text{-}17\) However, CRISPR/Cas9 has been demonstrated as an easy-handle, highly specific and efficient approach for editing the eukaryotic genome.\(^18\) As survivin plays vital roles in inhibition of apoptosis and cell death in leukemic cells, here we set out to explore the disruption of its gene, BIRC5 by CRISPR/Cas9. Herein, we demonstrated that knockout of BIRC5 by CRISPR/Cas9 in leukemic cells is technically feasible and efficient, also this gene-editing leads to induction of apoptosis and inhibition of cell growth. Moreover, our data show the therapeutic application of CRISPR/Cas9 for disruption of oncogenic BIRC5 in cancers including leukemia.

**Materials And Methods**

**Cell Culture**

The human erythroleukemia KG1 cell line and human promyelocytic leukemia HL60 were obtained from Pasteur Institute of Iran. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) containing 10% heat-inactivated (30 min, 56°C) fetal bovine serum (FBS) (Gibco, Langley, OK), 1% Glutamax (Gibco), 1% penicillin/streptomycin at 37°C and 5% CO\(_2\) in fully humidified incubator.

**Vector Construction And Expression**

CRISPR/Cas9 system was purchased from GeneCopoeia (Rockville, MD). To drive the expression of the Cas9 protein, Cas9 nuclease expression clone was specifically designed to have CMV promoter, a mammalian antibiotic resistance gene as a stable selection marker (Neo), and a reporter gene, mCherry fluorescent protein. Also, the negative control (Scramble) vector for pCRISPR-SG01 was constructed as the same as the sgRNA plasmid backbone which did not contain a sgRNA sequence. Three different sgRNAs (sgRNAa, sgRNAb, and sgRNAc) were then designed to construct the CRISPR/Cas9 to target human BIRC5. Briefly, the sequences of sgRNAa, sgRNAb, and sgRNAc are 5′ CCAGCCAGGGGACAAGTCG 3′, 5′ A CTTACATGGGTCTGTCATC 3′, and 5′ GGCCAGTCT CACCCGCTCCG 3′, respectively, which targeted exon 1 except for sgRNAb that generated an indel mutation in exon 2 of the gene. These sequences were separately inserted into the sgRNA expression cassettes of pCRISPR-SG01 vector containing the U6 promoter. All of the vectors were introduced by chemical transformation into the competent E.coli DH5α for cloning purposes using a selectable marker of Ampicillin. These strains were cultured on LB agar plates supplemented with 1 mg/mL Ampicillin at 37°C and subsequently in LB broth, liquid culture, containing Ampicillin in the 37°C shaking incubator at 180 rpm/min speed. Then, all of the plasmids were purified with EndoFree Plasmid Maxi Kit (Qiagen, Germantown, MD) according to the manufacturer’s protocol.

**Cell Transfection**

8 × 10\(^5\) KG1 and HL60 cells were dispensed in 6-well plastic tissue-culture plates containing 2 mL of fresh RPMI 1640. The cells were then transfected with either the Cas9- and three sgRNAs expressing plasmids or scramble DNA using Lipofectamine 3000 (Invitrogen Waltham, MA). For a single well of a 6-well dish, 2500 ng plasmid DNA, 5 µL of P3000 Reagent, 6 µL of Lipofectamine 3000 reagent, and 250 µL of OptiMEM were used, according to the supplier’s protocol. After 6 hrs, supplemented media consisting of 10% FBS and 1% penicillin/streptomycin was added to each well. To investigate co-transfection efficiency, cultures were visualized.
through fluorescence microscopy 48 hrs’ post-transfection by monitoring the expression of the reporter gene, mCherry.

DNA Extraction and PCR Amplification Assay

Genomic DNA (gDNA) was extracted using the PrimePrep™ Genomic DNA Isolation Kit (GeNet Bio, Daejeon, Korea) 48 hours after transfection, and quantification of gDNA was performed by NanoDrop spectrophotometer (WPA Biowave II, Cambridge, UK). The DNA region encompassing the CRISPR target site in BIRC5 was amplified with Pfu high fidelity DNA Polymerase (Vivantis Technologies, Kuala Lumpur, Malaysia) using the sense 5′- GACTACA ACTCCCGGCAACAC −3′ and antisense 5′– AAGGCA TGAGGCATCTTTACG −3′ primers. PCR was performed under the standard following conditions: 1 cycle initial denaturation of 3 mins at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C, with a final 10 mins at 72°C for post extension. Amplified PCR products were simply subjected to electrophoresis on 1.5% agarose gel prestained with DNA Green Viewer (Parstous, Iran) at 80 volts for 30–45 mins. The 868 bp fragment was then excised from the gel and purified using the AccuPrep™ Gel Purification Kit (Bioneer, Daejeon, Korea).

**Surveyor Mutation Detection Assay**

BIRC5 oncogene knockout was examined by the SURVEYOR Mutation Detection Kit (Integrated DNA technologies, USA) according to the producer’s instructions. Briefly, 400 ng of equal amounts of the test (transfected) and reference (untransfected) purified PCR products were mixed in a microtube for DNA duplex formation. Then, to promote heteroduplex formation, the PCR products heated and cooled slowly on a Bio-Rad C1000 thermocycler as follows: 1: 95°C for 10 mins; 2: 95°C to 85°C ramping at -2.0°C/s; 3: 85°C for 1 min; 4: 85°C to 75°C ramping at -0.3°C/s; 5: 75°C for 1 min; 6: 75°C to 65°C ramping at -0.3°C/s; 7: 65°C for 1 min; 8: 65°C to 55°C ramping at -0.3°C/s; 9: 55°C for 1 min; 10: 55°C to 45°C ramping at -0.3°C/s; 11: 45°C for 1 min; 12: 45°C to 35°C ramping at -0.3 °C/s; 13: 35°C for 1 min; 14: 35°C to 25°C ramping at -0.3°C/s; 15: 25°C for 1 min. After reannealing, the samples were immediately kept on ice and 1/10 of the total volume of each reaction mixture 1.5 M of MgCl₂ was added. Next, 1 µL of Surveyor Enhancer S and 1 µL of Surveyor Nuclease were added to the products followed by 60 mins incubation at 42°C. Then, stop solution was added in 1/10 volume of each product and digestion products clearly analyzed by 2% agarose gel. The indel percentage was calculated according to the gray value detected using ImageJ software (NIH, Bethesda, MD). The percentage of mutation was measured using the following formulas wherein a is the integrated intensity of the undigested PCR product and b and c are the integrated intensities of each cleavage product:

\[
    f_{\text{cut}} = \frac{b + c}{a + b + c} \\
    \text{Mutation~}\% = 100 \times (1 - \sqrt{1 - f_{\text{cut}}}).
\]

**RNA Extraction And RT-qPCR Assay**

Total RNA was extracted from cells using the YTA Total RNA Purification Mini Kit (Yekta Tajhiz, Tehran, Iran) 48 hrs after transfection, according to the supplier’s protocol. Complementary DNA (cDNA) was synthesized from 2 µg total RNA as a template by using random hexamer. Reverse transcription was performed using the RevertAid™ First-Strand cDNA Synthesis (Thermo Fisher Scientific, Germany) for 60 mins at 42°C followed by 70 mins at 5°C with RevertAid Reverse Transcriptase.

Quantitative Real-Time PCR (RT-qPCR) assay was carried out with Real Q Plus 2X Master Mix Green high ROX™ Kit (Ampliqon, Denmark). PCR amplification was performed to produce an amplicon of 249 bp in 10 µl final reaction volume with the SYBR Green detection method using the Step one plus Real-Time PCR System (Applied Biosystems, USA). The sequences of the primer sets were used, including BIRC5-F, 5′– CGCATCTCTA CATTCAAG –3′; BIRC5-R, 5′– ATGTTCCTCTCTCGT GAT –3′; GAPDH-F, 5′– AAGCTCATTCTCTGGATAT –3′; GAPDH-R, 5′–5″ CTTCCCTTGTGCTTGG –3′; and c are the integrated intensities of each cleavage product:

\[
    f_{\text{cut}} = \frac{b + c}{a + b + c} \\
    \text{Mutation~}\% = 100 \times (1 - \sqrt{1 - f_{\text{cut}}}).
\]

**Cell Viability Assay**

Cell viability was analyzed by the MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide)
viability assay as previously reported. In brief, the cells were seeded in 96-well plates in triplicate at a density of $5 \times 10^3$ cells per well, 48 hrs after transfection of cells with CRISPR/Cas9 vectors. Next, 20 µL of the colorimetric MTT reagent was directly added to the wells with a final concentration of 1 mg/mL. Then, the cells were incubated for 4 h at 37°C, the medium was discarded completely and 100 µL Dimethyl sulfoxide was added and shaken for 10 min. The optical density (OD) was measured at 570 nm using a microplate reader (Stat Fax, Palm City, FL). The absorbance of control cells was considered as 100%. Percent of cell viability was calculated as $= 100$-(test OD/control OD) x100.

**Apoptosis And Necrosis Assay**

Forty-eight hours after transfection, cells were harvested and apoptosis was analyzed as previously reported. The cells were stained for Annexin V and PI according to the manufacturer’s instructions. Briefly, cells were harvested, washed and incubated with Annexin V and PI for 15 min in dark place. Next, after washing with 300 µL binding buffer, cells were gently resuspended in 300 µL of binding buffer, kept on ice and subjected to FACS analysis (FACS Calibur, Beckman Dickinson, San Jose, CA) within an hour. Flow cytometry data were analyzed by FCS Express software (De Novo Software, Los Angeles, CA).

**Statistical Analysis**

Arithmetic means and standard deviations were calculated and statistical significance was defined as $P \leq 0.05$ using Student’s $t$-test.

**Results**

**The Design And Validation Of sgRNA Targeting BIRC5**

In the present study, we first retrieved the sequence of BIRC5 gene (NCBI Reference Sequence (RefSeq): NG_029069.1) and then performed the designation of sgRNAs. We designed three constructs to express sgRNAs of the CRISPR/Cas9 nuclease toolkit. All of these sgRNAs are in principle composed of 20 nt in length, preceded by the canonical trinucleotide 5’-NGG, the protospacer adjacent motif (PAM). The plasmid-based sgRNA expression constructs possess the U6 RNA polymerase III promoter, which, in turn, prefers a guanine (G) nucleotide as the first base of its transcript; thus, an extra G residue is added at the 5’ of the sgRNA. To minimize the off-target mutations, the DNA target site should entirely match the PAM motif as well as the 12 bp seed sequence closest to the PAM; the remaining bases are thought to be less important as their mismatches could be tolerated. In our study, sequences that contained the PAM motif inside exon 1 and/or 2 of BIRC5 were identified. Then, to determine whether or not candidate sequences were unique in the genome, they were analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The schematic features of DNA target sequences and their corresponding sgRNAs are depicted in Figure 1A and B.

**Optimizing Cas9/SgRNAs Delivery Of Leukemic Cells**

We next aimed at optimizing conditions for the co-transfection of Cas9/sgRNA expression plasmids leukemic cells via lipofectamine. For this purpose, we used reporter plasmids mCherry as a reporter gene in Cas9 and scramble vectors. As shown in Figure 1C, fluorescence micrographs of mCherry-transfected cells indicated that Lipofectamine 3000 is an appropriate reagent for delivering Cas9 or scramble in leukemic cells.

**Verification Of The Effect Of sgRNAs Targeting Human BIRC5 Gene**

To characterize the functionality of Cas9 nuclease, as well as their respective sgRNAs on BIRC5 gene, we used the SURVEYOR nuclease assay. This method is a mismatch-specific DNA endonuclease for indel mutations identification through the generation of double-strand breaks (DSBs) and subsequent NHEJ DNA repair mechanism. Genomic DNA was isolated from transfected cells and screened for the presence of site-specific gene modification by PCR. As shown in Figure 2, both of the duplex DNA strands were catalyzed at 3 bp upstream of the PAM. Moreover, our data show that PCR amplicon of 868 bp in BIRC5 gene was broken into several smaller fragments, where sgRNAa, sgRNAb, and sgRNAc were cleaved at their 176 bp, 625 bp, and 269 bp positions, respectively (Figure 2). According to these results, we found that all of sgRNAs successfully triggered site-specific cleavage in the BIRC5 gene locus.

**Evaluation Of Survivin Expression At mRNA Levels**

To confirm that BIRC5 gene was thoroughly disrupted in these leukemic cells, we next examined the expression of
survivin at mRNA level by RT-qPCR. We observed that survivin is highly expressed in these cells confirming the previous reports\(^\text{14}\) and disruption of \textit{BIRC5} by CRISPR/Cas9 leads to a significant reduction (30 fold) of survivin in these cells. However, the scramble did not show any significant difference in the expression of survivin in both cell lines (Figure 3).

The Effect Of CRISPR/Cas9-Mediated \textit{BIRC5} Knockout On Cells Survival And Apoptosis

Next, we examined cell proliferation in CRISPR/Cas9-mediated \textit{BIRC5} knockout HL60 and KG1 by MTT assay and found that the disruption of \textit{BIRC5} resulted in
Figure 2 CRISPR/Cas9-mediated cleavage at BIRC5 locus in AML cells. (Left) PCR detection and (Right) Surveyor assay of CRISPR/Cas9 activity in HL60 and KG1 cell lines. The numbers on the left represent the sizes of the DNA Ladder. The numbers at the bottom of the gel indicate mutation percentages measured by band intensities.

Figure 3 BIRC5 expression was quantitatively evaluated via RT-qPCR 48 h after cotransfection using sgRNAs, sgRNAb, sgRNAc and Cas9 vectors. Relative expression values were normalized assigning the value of the cells in control groups to 1.0. Error bars represent mean ± s.d. of biological replicates from one experiment (P<0.0001).
a significant decline of cell viability compared to control and scramble DNA (Figure 4).

Having shown that CRISPR/Cas9-mediated \textit{BIRC5} knockout reduces cell viability, we sought to examine if disruption of \textit{BIRC5} by CRISPR/Cas9 could lead to the induction of apoptosis. To do this, the cells were stained with Annexin V and PI, and the apoptosis was detected by flow cytometry. As demonstrated in Figure 5, CRISPR/Cas9-mediated \textit{BIRC5} knockout shows a remarkable induction of apoptosis in both cell lines. However, scramble plasmid did not induce apoptosis in these leukemic cells.

**Discussion**

The CRISPR/Cas9 technology has been employed on the genome manipulation, modification, and engineering in microorganisms, plants, animals, and humans for experimentally and therapeutically purposes. In the current study, we successfully disrupted the \textit{BIRC5} gene in two human leukemic cells and observed that this genome-editing toolkit is not only feasible and easy to handle but also capable of inducing apoptosis and cell death in these leukemic cells.

Previous studies clarified that \textit{BIRC5} gene is overexpressed in numerous malignancies such as leukemia and...
correlated with tumor progression and drug resistance.\(^9\) The most distinctive function of survivin is cell death regulation. This protein cannot directly interact with caspases; it associates with the X-linked inhibitor of apoptosis (XIAP) and represses the caspase-dependent apoptotic pathway. The emerging body of evidence indicates that survivin acts as anti-apoptotic protein, as well as the promotion of cancer cell invasion and migration, implying that survivin can be considered as a noticeable prognostic and metastatic factor in cancerous cells.\(^21\) Several lines of evidence made it apparent that high \textit{BIRC5} expression has been associated with substantially inferior, poor, and adverse clinical outcome in AML.\(^10,11,22,23\)

Previous studies have demonstrated that the isoforms (\(-2B, -Ex3\) and \(-3B,\) except survivin-2\(\alpha\)) of survivin are overexpressed in a human AML-M3 cell line, NB4, as well as bone marrow samples from patients. However, their expression was declined after arsenic trioxide (ATO) treatment as a front-line therapy.\(^10\) In 2017, Pazhang et al\(^11\) have demonstrated survivin was downregulated in another AML-M3 cell line, HL60 following treatment with embelin and celastrol, two anti-tumor agents, and leads to a considerable response improvement to chemotherapeutic agents. Accordingly, another study has revealed that pretreatment with survivin siRNA could synergistically sensitize leukemic cells U937 cells to anticancer drugs and induced apoptosis in these cells.\(^9\) Thus, survivin is a key player in leukemic cells survival and their resistance to chemotherapeutic regiments; therefore, we hypothesized that \textit{BIRC5} knockout with CRISPR/Cas toolkit would result in the induction of apoptosis and cell death in HL60 and KG1. Herein, for the first time, we employed the CRISPR/Cas9 gene knockout system to disrupt the \textit{BIRC5} gene in two human leukemic cells. It is obvious that the transfection of non-adherent cells such as leukemic cells is rather difficult than adherent cell line. However, we co-transfected three DNA plasmids into HL60 and KG1 cell lines by lipofectamine 3000. Thus, this novel non-viral-mediated gene-editing method has many advantages of clinical applications such as low cost and safety. In line with our observations, a recent study employed Cas9:sgRNA gene knockout system to disrupt the PDL-1 gene in human lymphocytes.\(^24\) In accordance with our data and previously published reports, we envision that designing of CRISPR/Cas9 toolkit to disrupt overexpressed genes such as survivin in leukemic cells is a feasible and effective approach. Consistently, we have recently reported that CRISPR/Cas9 nickase (Cas9n) is an alternative approach for knocking out the leukemic cells.\(^25\) Cas9n is a mutated form of Cas9 which stands in need of two hemiclease domain to create a double-stranded break at the target site.\(^25\) We envisioned that both CRISPR/Cas9 and CRISPR/Cas9n are feasible approaches to generate genetically modified human cells. However, on-target mutations of CRISPR/Cas9 are nearly twice that of CRISPR/Cas9n in HL60 cell lines, but there are equal in the KG1 cells. On the other hand, designing of sgRNAs for CRISPR/Cas9 is more feasible than CRISPR/Cas9n. Altogether, both systems are appropriate methods for editing or knocking out a gene/ oncogene. However, each system might be more suitable for a certain type of cells or cancer.

Furthermore, we delineated an inverse correlation between survivin protein levels and apoptosis in leukemic cells. Having knocked out cells with CRISPR constructs, we detected an increasing number of programmed cell death, confirming the survivin invaluable function in cancer development, cell survival, and proliferation. Our data are in close agreement with previous ones which unraveled survivin/\textit{BIRC5} mechanistic events was the pivotal and ubiquitous nature of tumor progression and clinical manifestation, consequently emerged as an attractive therapeutic target in cancer treatment.\(^26–29\)

Collectively, this study strengthens the idea that cationic lipid delivered efficiently Cas9: sgRNA into leukemic cells and that CRISPR/cas9 editing gene could be an appropriate method for disrupting the overexpressed gene such as \textit{BIRC5} which play an important role in the pathogenesis of cancers including leukemia. Further investigations of employing CRISPR/cas9, particularly in an animal model and preclinical studies, may pave the path for personalized medicine and cell therapy.

**Author Contributions**

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

**Disclosure**

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