



Survivin Gene Disruption via CRISPR/Cas9 Induces Apoptosis Through Down-regulation of FBXO5 and RRM2 in Prostate Cancer Cell Line PC3

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Abstract

Objectives: Prostate cancer (PC) is a complex and heterogeneous disease that arises from both genetic and epigenetic alterations. Survivin acts as a bifunctional controller of apoptosis restraint and is up-regulated in numerous human cancers involving PC. CRISPR/Cas9 was illustrated as a profoundly specific and effective method for altering genes involving oncogenes.

Materials and Methods: Colony polymerase chain reaction (PCR) was performed to identify the transformed colonies. Plasmid purification was performed from desired colonies due to the manufacture plasmid extraction kit protocol. A plasmid involving Cas9 and sgRNAs was co-transfected into PC3 cells using lipofectamine 3000. A vector with no cloned gRNA was utilized for scrambling. The efficacy of transfection and the expression levels of ribonucleotide reductase (RNR) small subunit M2 (RRM2) and FBXO5 were identified by quantitative reverse transcription-PCR. Cell proliferation and apoptosis were assayed by XTT assay and Annexin V-PE/7-AAD, respectively. Data were assayed by utilizing one-way ANOVA and Tukey's test using SPSS (version 20, USA), and $P < 0.05$ was considered statistically significant.

Results: The results revealed that lipofectamine 3000 is an efficient approach for delivering on the CRISPR/Cas9 system in PC3 cells. The knocked out of survivin by the CRISPR/Cas9 significantly decreased the proliferation and induced apoptosis of transfected PC3 cells compared to the scrambling vector. Finally, CRISPR/Cas9 systems significantly down-regulated the expression levels of RRM2 and FBXO5 at mRNA levels (fold change 0.406, $P = 0.0002$).

Conclusions: In general, targeting survivin by CRISPR/Cas9 led to the down-regulation of RRM2 and FBXO5, as well as the induction of apoptosis in PC3 cells. Thus, more research using further PC cell lines and primary cells is necessary.

Keywords: Prostate cancer, Survivin, Apoptosis, Crispr/cas9

Introduction

Prostate cancer (PC) is considered as one of the foremost usual malignancies in men and the moment driving cause of cancer-associated mortality in developed countries. In addition, it could be a complex and heterogeneous disease which emerges from both hereditary and epigenetic changes (1). The development of human PC is distinguished by intraepithelial neoplasia, adenocarcinoma androgen-dependent, and adenocarcinoma androgen-independent or castration-resistant steps. Previous investigations show that the abnormal regulation of many genes is important in cancer development. Although there were critical progresses within the early discovery and treatment of PC, the key determinant of lethality within the infection remains a major clinical challenge (2). Hence, it is critical to absolutely dismember principal atomic components directing PC and engage the personalized medication technique through innovatively progressed apparatuses. Similarly, programmed cell death in cancer seems to be a reasonable procedure for restorative methodologies in future (2).

A deep note of survivin in PC cells versus its absence in the common secretory epithelium of the prostate indicates the biological significance of the present oncoprotein in PC biology (3). Further, a strong correlation was identified among high levels of survivin expression and tumor clinicopathological characteristics, as well as increased resistance to treatment in various cancers (4-6).

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and structurally has a BIR domain, well-conserved N-terminal ~70 amino acids, which handle the zinc ion by cysteine and histidine residues (7,8).

A high level of survivin expression leads to the inactivation of the receptors of tyrosine kinase, including the receptor of epidermal growth factor 1, insulin-like growth factor-1, insulin-like growth factor-1 receptor, and Erb-B2 receptor tyrosine kinase 2 (ERBB2), as well as several cell signaling pathways such as STAT, mTOR, HIF-1, PI3K/Akt, and MEK/MAP followed by survivin (8,9).

The nucleotide metabolism enzyme, namely, ribonucleotide reductase (RNR) is necessary for DNA

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Key Messages

- ▶ Prostate cancer is the most common malignant cancer among men. Using novel gene therapy-based methods for treatment of cancers has gotten increasing attention during the recent years.
- ▶ This study was aimed to evaluate the inhibitory effect of MAGE-A11 gene by CRISPR / Cas9, as an important oncogene involved in the pathophysiology of prostate cancer.

synthesis and DNA repair by creating dNTP. RNR activity is coordinated with cell cycle progression to maintain the fine balance between dNTP production and DNA replication. RNR has three subunits including RRM1 and RRM2B, which are constant during the cell cycle, and RRM2 levels fluctuate during the cell cycle. The overexpression of RRM2 leads to the induction of lung cancer in mice. Its high expression is associated with several cancers (10,11). It was shown that RRM2 overexpression is related to surviving expression in lung carcinoma (12). Moreover, it was reported that FBXO5, which promotes cell proliferation, tetraploidy, and genomic instability, is overexpressed in malignant tumors rather than benign tumors. For example, FBXO5 overexpression was associated with poor outcomes of PC (13).

Given that a strong relationship was detailed between hoisted survivin expressions and clinicopathological indicators of several cancers, a few pharmacological and hereditary approaches were utilized to interfere with survivin function, involving antisense oligonucleotides, ribozymes, and little interferometer RNAs (siRNAs), but none of them can permanently silence it (14,15). Accordingly, the utilization of “clustered regularly interspaced short palindromic repeats” (CRISPR)/“CRISPR-associated qualities 9” toolkit to knockout this prominent protein may pave the way for understanding the exact mechanism of survivin (15-17).

CRISPR/Cas9 has been shown to be an easy, profound, and efficient way to modify the genome of eukaryotic. Since it plays a crucial part in the hindrance of apoptosis in PC cells (6,18), this study focused on investigating the effects of survivin knockout on apoptosis and cell proliferation.

Materials and Methods

Cell Culture

The Human PC Cell Line PC-3 was bought from Pasteur Institute of Iran, Tehran. The cells were cultured in RPMI 1640 medium involving 10% fetal bovine serum (Gibco,

England, London) plus 1% penicillin/streptomycin (Sigma) in a fully humidified atmosphere at 5% CO₂ and 37°C until achieving the exponential growth phase and 70%-80% confluence.

Vector Construction, Target Design, and Cloning

Two sgRNAs were planned by utilizing crispr.mit.edu and crispor.tefor.net, and the oligos were synthesized by Microgen in Seoul, South Korea. The applied sgRNAs arrangements for knocking out the survivin gene are outlined in Table 1. The PCG-eCas9-GFP-U6-gRNA vector (Addgene 79145) was digested with the BbsI enzyme for gRNA cloning. gRNA arrangements using the T4 ligase enzyme were embedded into the straight vector, and the ligated product was introduced through thermal shock into the competent *Escherichia coli* DH5α for cloning purposes by employing a selectable marker of ampicillin. These strains were cultured in Luria-Bertani (LB) agar involving 100 µg/mL of ampicillin at 37°C. Consequently, they were grown in LB broth involving ampicillin in a 37°C shaking incubator at 200 rpm.

The colony polymerase chain reaction (PCR) was performed to identify the transformed colonies. Plasmid purification was conducted from desired colonies according to the protocol of the plasmid extraction kit. Sequencing was performed to confirm the correct insertion of sgRNA oligomers into the plasmid.

Cell Transfection

PC3 cells were seeded in six-well plates at a thickness of 5×10^5 cells/well and developed to 70% juncture, where the plasmid containing sgRNAs was co-transfected to the cells by lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The PCG-eCas9-GFP vector without any cloned gRNA was utilized for the scramble. According to the protocol of transfection, 5 µL of Lipofectamine 3000 reagent, 6 µL of P3000 reagent, 3000 ng of plasmid DNA, and 250 µL of Opti-MEM were added to each well, and the supplemented media involving 10% FBS and 1% penicillin/streptomycin were added to each well after 6 hours.

Assay for Genome Modification

The transfected cells were collected. Then, the genome of the transfected cells was extracted using a genomic DNA extraction kit (Macherey Nagel, Germany). To detect genome modifications at the targeted region, the PCR was performed using primers flanking the targeted region. The corresponding primers are provided in Table 2. The PCR product was loaded on 1% agarose gel, and the expected band was visualized by UV.

Table 1. The Sequences of Primers Used for Investigating the Colony PCR

Primer	Sequence 5'→3'	TM	Product Size (bp)
Forward	5'- CGATACAAGGCTGTTAGAGAG -3'	60	220
Revers	5'- AAACACGGGTCCCGCGATTCAAATC-3'		

Note. PCR: Polymerase chain reaction.

Table 2. Primers Used for Flanking the Targeted Region

Primer	Sequence 5'→ 3'	TM	Product Size (bp)
Forward	5'- ACTACAACCTCCCGGCACAC -3'	60	923
Revers	5'- GGGTCTGCTGATGATTTCTG -3'		

Cell Viability

Cell viability was assessed by Cell Checking Kit-8 (CCK-8 viability assay). The cells were seeded on a 96-well plate at a thickness of 10^4 - 10^5 cells/well in 100 μ L of culture medium in triplicate, and then co-transfected with a plasmid containing sgRNAs and scrambling. Twenty-four and forty-eight hours after transfection, 10 μ L of the CCK-8 solution was included in each well of the plate and brooded for 1-4 hours within the incubator. Moreover, the absorbance was measured at nm by employing a microplate per user set at 450 nm. The absorbance of untreated cells was considered as 100%. Rate development restraint was calculated using rate of restraint = $100 - [(test\ OD / control\ OD) \times 100]$ (19).

The qRT-PCR Assay

Total RNA was extricated from cells by utilizing NucleoSpin1RNA Pack (Macherey-Nagel) according to kit instructions. A total of 500 ng RNA was applied for cDNA synthesis. cDNA was synthesized from total RNA using the cDNA Synthesis Kit (Takara, Japan) based on the protocol. The survivin, RRM2, and FBXO5 genes were expressed by the qPCR within the Rotor-Gene 6000 system (Corbett Research, Mortlake, NSW, Australia). The primers are presented in Table 3. The applied cycle conditions for all genes involved an initial step of 95°C for 30 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and extension at 72°C for 45 seconds with one cycle melt curve stage of 95°C for 5 seconds, and finally, 95°C for 15 seconds. The relative quantification of gene expression was calculated using the comparative Ct method with equation after $2^{-\Delta\Delta C_t}$ normalization of the target gene mRNA level with an endogenous housekeeping β -actin gene.

Apoptosis Assay

The cell apoptosis was evaluated by Annexin V-PE/7-AAD Apoptosis Detection Kit. The cells were seeded on a 6-well plate at a thickness of 250 000 cells/well in 2 mL of culture medium. At that point, they were co-transfected with the plasmid containing gRNAs. The cells were centrifuged and washed in PBS 48 hours after transfection. Next, they were resuspended in 400 μ L official buffer and recolored with 5 μ L of annexin V-PE and 5 μ L of 7AAD for 15 minutes within the dim, and eventually analyzed by flow cytometry.

Statistical Analysis

The results are detailed as the mean \pm standard error of the mean of three replicates per group. Data were analyzed by one-way ANOVA and Tukey's test using SPSS (version 20, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Confirmation and Accuracy Insertion of sgRNA Oligomers into the Plasmid

First, colony PCR was performed to ensure that the sgRNA oligos are on the plasmids of the accession product. Sequencing was conducted to confirm the correct insertion of sgRNA oligomers into the plasmid. SangRNA sequencing approved the sgRNA in the plasmid, along with its accuracy (Figure 1).

Transfection Efficiency PC3 Cell Line by PCG-eCas9-GFP

PC3 cells were seeded in six-well plates at a density of 5×10^5 cells/well and grown to 70% confluence with the plasmid involving sgRNAs co-transfected to cells using Lipofectamine 3000. Figure 2 depicts PC3 cells efficiently

Table 3. The Sequences of the Applied Primers for Investigating the Expression Level of Survivin, RRM2, and FBXO5 Genes

SgRNAs and Primer Sequence			
Primer	Sequence 5'→ 3'	TM	Product Size (bp)
Survivin- Forward	ACCACCGCATCTCTACATTC	60	132
Survivin- Revers	AGAAGAAACACTGGGCAAG		
Guid 1 for survivin gene	Sense: CACCGATTGAATCGCGGGACCCGT Antisense: AAACACGGGTCCCGCGATTCAAATC	-	-
Guid 2 for survivin gene	Sense: CACCGACTTACATGGGGTCTGCATC Antisense: AAACGATGACGACCCCATGTAAGTC	-	-
FBXO5- Forward	AAGCAATACAAAGAGTTACCG	56	159
FBXO5- Revers	CACCTTGATTGGATAACTTGG		
RRM2- Forward	AGTCAGTTGGTGCCAGATAG	57	123
RRM2- Revers	TCCTTGCCCTGAGAGATTC		
β -actin- Forward	AGATCATTGCTCCTCTGAG	56	162
β -actin- Revers	CTAAGTCATAGTCCGCCTAG		

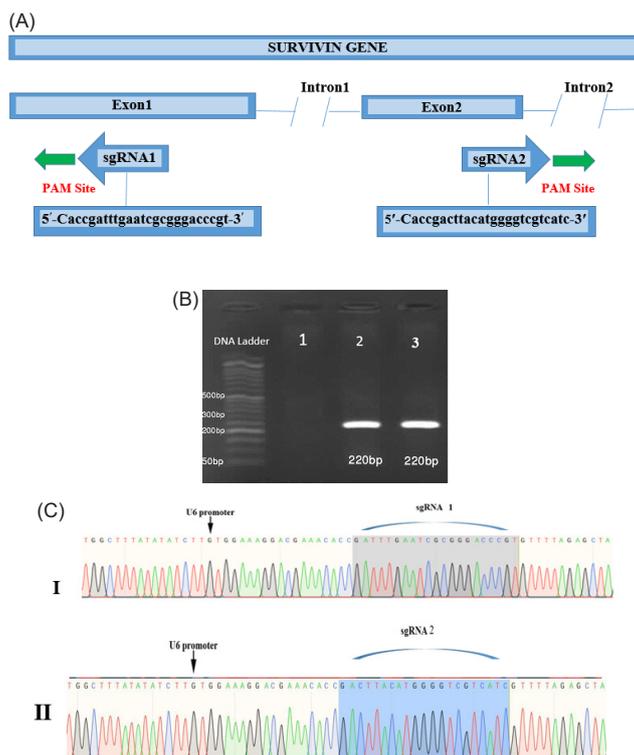


Figure 1. Illustration of the Designed CRISPR/Cas9 for the Knockout of the Survivin Gene.
Note. PCR: Polymerase chain reaction. **A.** Schematic of the exon 1,2 of Survivin gene and location of gRNAs. **B.** The results of colony PCR confirmed the correct insertion of sgRNA oligos in the vector: 1, in that negative control sample uploaded. 2 and 3 indicate that the transformants were positive for plasmid fusion with sgRNA oligomers. **C.** SangRNA sequencing approved the sgRNA in the plasmid and its accuracy. **I.** sgRNA sequencing for sgRNA1 and **II.** sgRNA sequencing for sgRNA2 Survivin gene.

transfected with a plasmid containing sgRNAs as detected by fluorescent microscopy and flow cytometry.

Knocking out by CRISPR/Cas9 Resulted in Reduction in the Expression Level of Survivin

Genomic PCR displayed the deletion of the survivin gene in the transfected cell compared to the control group. After the analysis of survivin knockout on the DNA level, the expression level of survivin in transfected PC3 cells was assessed by the qPCR. qRT-PCR data were assayed by utilizing the $\Delta\Delta CT$ procedure, and it was found that the

survivin expression level in transfected cells significantly reduced compared to the scramble and control group (fold change 0.293, $P < 0.0001$, Figure 3B).

Knocking out of Survivin by CRISPR/Cas9 Significantly Decreased Proliferation and Induced the Apoptosis of Transfected PC3 Cells

The effects of survivin knockout on the proliferation of transfected PC3 cells were analyzed using CCK8 Assay Kit after 48 hours. Our data indicated that cell proliferation in control and negative control (scramble) groups were nearly

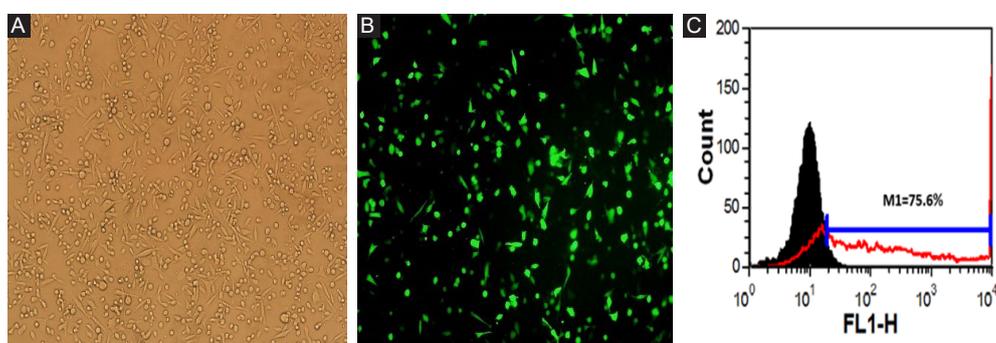


Figure 2. Assessment of Transfection Efficiency of PC3 Cell Line by PCAG-eCas9-GFP.
Note. PC3 cells were seeded in six-well plates at a density of 5×10^5 cells/well and grown to 70% confluence, the cells were cotransfected using Lipofectamine 3000. **A.** Image of control pc3 cells with contrast phase microscope. **B.** Fluorescent microscopy images of pc3 cells transfected after 48 hours. **C.** The cells were assayed by utilizing flow cytometry 48 hours after transfection with PCAG-eCas9-GFP plasmid, and flow cytometry showed more than 75.5% transfected cells.

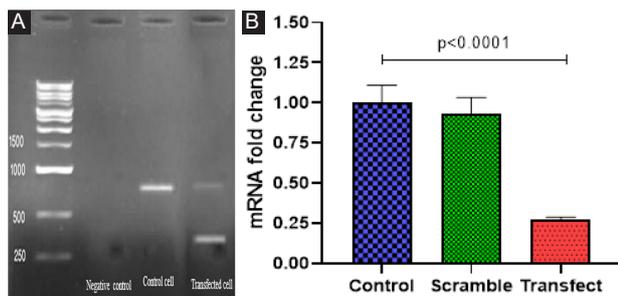


Figure 3. Knocking out of Survivin by CRISPR/Cas9 Resulted in Reduced **A.** Genomic PCR depicting a deletion of the Survivin gene in the transfected cell (band at 393 bp) compared to the control cell (band at 923 bp) and **B.** Survivin expression of Survivin decreased significantly compared to control and scramble ($P < 0.0001$).

the same although PC3 cells transfected with sgRNA/Cas9 indicated a drastic decline in proliferation. Furthermore, this study investigated whether knocking out survivin could result in the induction of apoptosis. In this regard, PC3 cells were co-transfected with a plasmid containing sgRNAs, and then were stained with annexin PI and 7, 48 hours after transfection, and finally, analyzed by flow cytometry. Transfected PC3 cells revealed significantly increased apoptosis compared to scramble and control groups ($P < 0.05$, Figure 4B).

Effect of Knocking out the Survivin Gene on the Expression Levels of RRM2 and Fbxo5

The expression level in survivin-knocked out PC3 was examined since RRM2 was shown to be associated with surviving expression. As illustrated in Figure 5A, RRM2 significantly decreased in survivin-knocked cells compared to the scrambling vector (fold change 0.406, $P = 0.0002$). Moreover, it was found that knocked-out survivin resulted in decreased expression of Fbxo5 (fold change 0.419, $P = 0.0001$) instead of but not scramble (Figure 5B).

Discussion

The CRISPR/Cas9 innovation was utilized on the genome control, alteration, and building in microorganisms,

creatures, and people for tentative and restorative goals. In the present research, the survivin gene in a human PC cell line (PC3) was effectively disturbed and the results revealed that this genome-editing toolkit is not only feasible and simple but also capable of actuating apoptosis and cell death in the PC. Additionally, it resulted in the down-regulation of RRM2 and Fbxo5, which are key players in the development of PC cells. Previous studies have indicated the up-regulation of the *BIRC5* gene in many human cancers (i.e., prostate, hepatocellular, and ovarian cancers) which is associated with inhibiting apoptosis and increased cell proliferation (6,19). The regulation of apoptosis is one of the important functions of survivin. Survivin indirectly inhibits caspase via X-IAP that leads to the inhibition of the caspase-dependent apoptotic pathway (20,21).

Likewise, survivin plays an important role in the progression of malignancy and metastasis by activating the MMPs (22). Moreover, the up-regulation of survivin was established in PC and human prostate cell lines involving PC3, DU-145, and LNCaP, as well as its role in tumor progression, metastasis, and treatment resistance (3).

Jiang et al indicated that surviving knock-down by small hairpin RNA (ad5-SVV) in A2780/CP cells (ovarian cancer cells) was correlated with increased apoptosis, inhibited cell proliferation, and invasion via the down-regulation of proliferating cell nuclear antigen and the overexpression of MMP-2 plus the high expression of caspase-3 (23). The other study indicated that pretreatment with survivin RNA interference (RNAi) could block cell proliferation and induce apoptosis in p53-dependent response (manner) in HeLa cells (24). In the current study, the CRISPR/Cas9 toolkit was employed to disrupt the survivin gene in PC3 cells. Consistent with our results, several studies showed that CRISPR-Cas9 is profoundly effective in mammalian gene ablation (25,26). Based on the results of this study, knocking out survivin by the CRISPR system alarmingly raised apoptosis and resulted in approved valuable survivin function in cancer progression.

The accumulating evidence indicates that RRM2

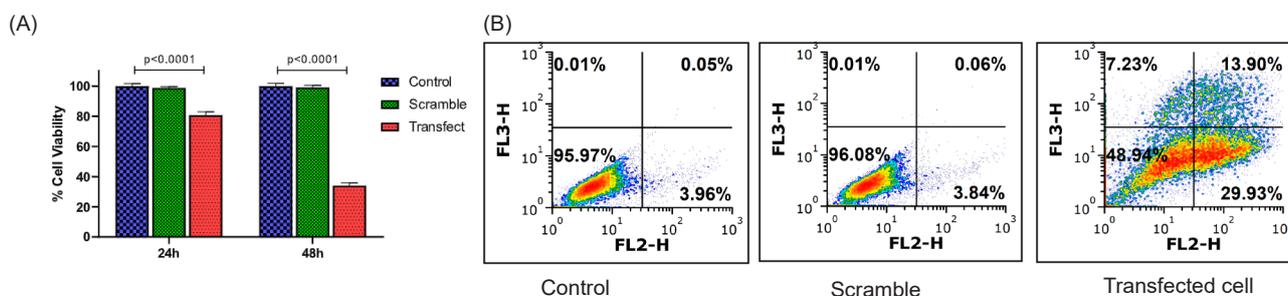


Figure 4. Targeting of Survivin Leading to Inhibition Proliferation and Induction of Apoptosis in Transfected PC3 Cells.

Note. **A.** The proliferation of PC3 cells was assayed by utilizing CCK-8 test 24 and 48 hours after transfection. Transfected PC3 cells revealed significant decreases in proliferation compared to scramble and control groups. **B.** Cells were stained with annexin PI and 7-aad 48 hours after transfection, and then were assayed via flow cytometry. Transfected PC3 revealed significantly increased apoptosis in comparison to scramble and control groups.

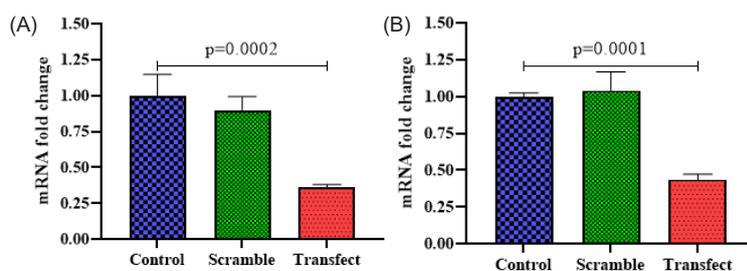


Figure 5. Effect of Knocking out of the Survivin Gene on the Expression Levels of RRM2 and Fbxo5 in the PC3 Cell Line by qRT-PCR 48 Hours After Transfection. *Note.* qET-PCR: Quantitative real-time polymerase chain reaction. **A.** RRM2 expression level remarkably reduced in survivin-knocked cells compared to the scramble vector and control ($P=0.0002$). **B.** Expression level of Fbxo5 significantly decreased in survivin-knocked cells in comparison to the scramble vector and control ($P=0.0001$).

is essential for DNA synthesis and DNA repair by producing dNTPs (27). It is frequently overexpressed in various cancers, including PC which is associated with chemoresistance, enhances cell invasion via the activation of the Ras/Raf signaling pathway, the up-regulation of MMP, and apoptosis inhibition (28,29). RRM2 upregulation was significantly related to the overexpression of survivin and DNA methyltransferase 1, which anticipated poor prognosis in gastric cancer patients (30). It was observed that knocking out survivin by CRISPR/Cas9 significantly downregulates RRM2 expression, indicating that surviving molecular pathway is associated with RRM2. Moreover, the up-regulation of FBXO5 was detected in several cancers, including PC, associated with chemoresistance and poor prognosis (31). Furthermore, the findings of the current research demonstrated that the disruption of survivin by CRISPR/Cas9 significantly decreased the expression level of Fbxo5. Although the signaling pathways of RRM2 and Fbxo5 were not evaluated in this study, it seems that these two cancer prognostic markers are somehow linked to the signaling pathway of survivin. Thus, further studies are required to find the possible cross-signaling of these molecules.

Collectively, targeting survivin by CRISPR/Cas9 induces apoptosis and slows the growth of PC, suggesting that CRISPR/Cas9 is a viable treatment intervention for the overexpression of tumors.

Conclusions

In general, it was shown that the knock-out of survivin via CRISPR/Cas9 in prostatic neoplasm cells is technically possible and efficient. Moreover, this gene modification resulted in the induction of apoptosis and reduced cell growth. Further, our result indicated the therapeutic application of CRISPR/Cas9 for the disruption of the up-regulation gene such as survivin, which plays an important role in the pathogenesis of cancers including PC. Further research while employing CRISPR/Cas9, mainly in an animal model and clinical trials, may pave the way for cell therapy.

Authors' Contribution

AJ and LF contributed to conception and design. LF, SF and FS

contributed to all experimental work, data and statistical analysis, and interpretation of data. AJ was responsible for overall supervision. LF drafted the manuscript, which was revised by AJ. All authors read and approved the final manuscript.

Data Sharing

All generated or analyzed data in this study are available upon reasonable request.

Conflict of Interests

The authors declare that they have no competing financial interests and nothing to disclose.

Ethical Issues

The present study was approved by the Ethics Committee of Kurdistan University of Medical Sciences, Sanandaj, Iran (code: IR.MUK.REC.1396/106).

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