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Original Article

The Disruption of Mage–11 Gene via CRISPR/Cas9 Method Induced Apoptosis in the *in vitro* Model of Prostate Cancer

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Abstract

Backgrounds and aims: Prostate cancer is the most common malignant cancer among men and is the second deadliest cancer in men after lung cancer. Understanding the molecular mechanisms involved in development and progression of prostate cancer is essential to improve both diagnostic and therapeutic strategies in this regard. In addition, using novel gene therapy–based methods for treatment of cancers has gotten increasing attention during the recent years. Accordingly, this study was aimed to evaluate the inhibitory effect of MAGE–A11 gene, as an important oncogene involved in the pathophysiology of prostate cancer *invitro* model. The study was also aimed to evaluate the downstream genes related to MAGE–A11.

Materials and Methods: First, MAGE–A11 gene was knocked out in PC–3 cell line using "Clustered regularly interspaced short palindromic repeats" (CRISPR)/ "CRISPR–associated genes 9" (CRISPR/Cas9) method. Next, the expression levels of MAGE–A11, survivin and Ribonucleotide Reductase Small Subunit M2 (RRM2) genes were determined by quantitative polymerase chain reaction (qPCR) technique. The levels of proliferation and apoptosis were also analyzed in PC–3 cells using CCK–8 and Annexin V–PE/7–AAD assays.

Results: The results showed that the disruption of MAGE–A11 by CRISPR/Cas9 method significantly decreased proliferation ($P<0.0001$) and enhanced apoptosis ($P<0.05$) in PC–3 cells compared to control group. Moreover, the disruption of MAGE–A11 significantly down regulated the expression levels of survivin and RRM2 genes ($P<0.05$).

Conclusion: Our results demonstrated that knocking out MAGE–11 gene by CRISPR/CAS9 technique could efficiently inhibit cell proliferation and induce apoptosis in PC3 cells. Survivin and RRM2 genes might also participated in these processes.

Keywords: Prostate Cancer, Apoptosis, CRISPR/Cas9, Mage–A11.

Introduction

Prostate cancer (PC) is the most frequently diagnosed neoplasia and the second most frequent malignancy (after lung cancer) in men worldwide[(1)]. The Global incidence of PC was estimated 1,300,000 new cases for 2018 and it was predicted that the incidence will reach 2,300,000 new cases until 2040[(2)]. The exact etiology of prostate cancer is still unknown. However, certain factors such as age, race and genetic background were shown to increase the PC development risk. Among these factors, Age is the most remarkable risk factor for prostate cancer.In fact, 80% of all prostate cancers are diagnosed in men over 60 years old and 95% of the men who die from the disease fall in this age group[(3)]. Accumulating evidence suggests that mutations and aberrant expression of certain genes are involved in the pathogenesis of prostate cancer.

Mutations and aberrant expression of specific genes are involved in the pathogenesis of prostate cancer

Current standard therapies, such as androgen deprivation therapy (ADT) and chemotherapy, that may provide temporary relief, are not definite cures.

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Recurrence, resistance to the treatment, tumor expansion, and metastasis are the main challenges of PC treatment that must be addressed [4]. In this regard, it is necessary to use molecular methods to identify and regulate the roles of biomolecules that are involved in the development and the progression of PC. Concurrent targeting of several components of survival, proliferation and apoptosis pathways in cancer cells via gene therapy–based approaches appears to be a prospective method for future therapeutic strategies. Over the last two decades, a variety of molecular and cellular technologies have been used to interfere/amplify molecular targets/pathways (e.g. short hairpin RNA (shRNA)[5], zinc–finger nucleases (ZFNs), and transcription activator–like effector nucleases (TALENs). However, most of these techniques have restrictions such as temporary inhibition of the gene function, off–target effects, and low efficiency of transfection to eukaryotic genomes. In recent years, a novel interesting method was introduced called "clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR–associated genes 9" (Cas9). This method, abbreviated as CRISPR/Cas9, have extremely been used in the genetic modification[6]. CRISPR / Cas9 has been indicated to be an easy, profound and efficient way to modify the eukaryotic genome[7] .

Numerous studies showed that an increase in the expression of MAGE antigens was associated with poor prognosis and increases in cancer progression hallmarks, including tumor growth, metastasis and resistance to chemotherapy[8]. These features make MAGE antigens potential interesting targets for gene therapy–based therapeutic approaches. MAGE antigens belong to the family of cancer/testis antigens (CTAs). The members of the human MAGE family Can be classified into two groups based on their tissue expression pattern: Type I MAGEs (A, B, and C), which are mainly expressed in tumor tissues, and Type II MAGEs (D, E, F, G, H, L, and necdins), which are be expressed in various normal tissues[9–11]. In recent years, various MAGE–A proteins have been demonstrated to regulate key factors of cancer–related pathways by targeting proteins such as the tumor–suppressor p53, survivin, Mdm2, PML–IV, E2F1, and AMPK[12–16].

MAGE–A11 is an oncogene, which is aberrantly expressed in many types of tumors, including ovarian, breast, and prostate cancers [17, 16, 18]. This MAGE is correlated with poor prognosis and progression of tumor. Up–regulation of MAGE–A11 affects various functions of tumor cells such as cell proliferation, apoptosis and metastasis.

Previous studies demonstrated that MAGE–A11 is a co–regulator of the Androgen Receptor (AR) and it is overexpressed during the progression of prostate cancer. This MAGE augmented the growth of prostate tumor via AR signaling and RB–related proteins. However, to our best of knowledge, its tumorigenicity via pathways independent of the androgen receptor (AR) is still unclear [5].

Nardiello et al (2011) reported that the downregulation of MAGE–A was associated with a reduce in the expression level of survivin in proliferating myeloma cells, which in turn led to an increase in apoptosis of the cells, and inhibition of proliferation via a p–53 dependent mechanism[15]. survivin is member of the inhibitor of apoptosis proteins (IAPs) family. it is highly expressed in many types of cancers including prostate cancer[19, 20], hepatocellular[21], and ovarian cancers[22]. Survivin is associated with inhibition of apoptosis, increased cell proliferation, tumor progression, and chemotherapy resistance.

Additionally, RRM2 is another important gene, which its overexpression is correlated with increased cell proliferation, malignancy and poor prognosis in prostate tumor[23]. RRM2 and RRM1 are two proteins, which together compose an enzyme called ribonucleotide reductase (RNR). RNR is essential for DNA synthesis and DNA repair by participating in production of dNTP[24].

According to the literature above, the present study aimed to evaluate the effect of knock outing of MAGE–A11 gene on the proliferation and the apoptosis of PC3 cell line (a prostate tumor–derived cell line) using CRISPR/Cas9 technique. Additionally, the expression level of survivin and RRM2 genes were evaluated as key players in cell proliferation and tumor progression of prostate cancer.

Materials and methods:

Cell culture

The human androgen–independent prostate cancer cell line, PC–3, was purchased from Pasteur Institute of Iran, Tehran. To reach the exponential growth phase, the cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated (30 min, 56°C) fetal bovine serum (FBS) (Gibco, England) and 1% penicillin/streptomycin (Sigma, Germany) in a fully humidified atmosphere at 5% CO2 and 37°C until 70–80% confluency.

Vector construction, target design, and cloning

Two sgRNAs were designed using online tools (crispr.mit.edu and crispor.tefor.net) and synthesized by Macrogen (Seoul, South Korea). The sgRNAs' sequences, used for knocking out the MAGE–A11 gene, are illustrated in table 3. In order to clone sgRNAs, PCG–eCas9–GFP–U6–gRNA vector (with ampicillin resistance gene as a selection marker) (Addgene, 79145) was linearized

with BbsI restriction enzyme (Thermo Fisher Scientific, United States). Then the sgRNAs were inserted into the vector using T4 ligase enzyme. Next, for amplification of the vector, it was introduced through thermal shock transformation into the competent E. coli DH5α. This bacterial strain was cultured in Luria–Bertani (LB) agar plates supplemented with 100 µg/ml of ampicillin at 37 °C. Subsequently, the transfected clones were screened by colony PCR technique. Then, they were cultured in LB broth, a liquid culture involving100µg/ml ampicillin in a 37 °C shaking incubator at 200 rounds per minute. Thereafter, the amplified vectors within the strain were extracted using Plasmid Mini Kit (Qiagen, Germany), according to the protocol of plasmid extraction kit. Next, sequencing was performed to confirm that the sgRNAs were inserted into the correct position on the vector.

Cell transfection

PC–3 cells were seeded in 6–well plates at a density of 5×10^5 cells/well and grown to 70% confluency. Then, the cells within the separated wells were considered as three experimental groups as following:

1) PC–3 cells without transfection (Control group)
2) PC–3 cells transfected with the sgRNA–containing vectors (Treatment group)
3) PC–3 cells transfected with the null vectors (Scramble group)

For transfection, each single well of the 6–well dish received a mixture of 3000 ng of the vector, 6 µl of P3000 Reagent (Invitrogen, USA), 5 µl of Lipofectamine 3000 (Invitrogen, USA), and 250 µl of Opti–MEM (Thermo Fisher Scientific, United States) in accordance to the protocol of transfection. Six hours after the transfection, complete culture medium containing 10% FBS and 1% penicillin/ streptomycin was added to each well. The cells were incubated for 24 or 48 hours, occasionally.

Genomic PCR

Genomic DNA of transfected cell was extracted utilizing Genomic DNA extraction kit (Macherey–Nagel, Germany). The extracted genomic DNA samples were amplified using PCR technique. Then, the PCR products were run on agarose gel.

Cell proliferation assay

Cell proliferation was assessed using Cell Counting Kit–8 (CCK–8) (Abcam, Switzerland). The Cells were seeded on a 96–well plate at a density of 10^4 cells/well in 100 µl of culture medium and grown to 70% confluency Next, the cells were categorized and transfected (similar to the section "2.3. Cell transfection" with appropriate amounts of the reagents). Then, 24 and 48 hours after transfection, 10 µl of the CCK–8 solution was directly added to each well, incubated for 1–4 hours at 37°C, and the optical density (OD) was measured using a microplate reader set at 450 nm. The absorbance of control cells was considered as 100%. The rate of growth inhibition was calculated as following: Percent of growth inhibition= 100– [(test OD/ control OD)x 100] [25].

The quantitative real–time PCR assay

Total RNA was extracted from cells utilizing NucleoSpin1RNA Kit (Macherey–Nagel), accordance to the kit instructions. Then, 500 ng of the total RNA was used for cDNA synthesis. cDNA was synthesized from total RNA using the cDNA Synthesis Kit (Takara, Japan) according to the supplier's protocol. Expression levels of MAGE–A11, survivin and RRM2 mRNAs were determined by qPCR using Corbett Rotor-Gene 6000 system (Corbett Research, Mortlake, NSW, Australia). The corresponding primers are shown in table 1.

The cycle conditions used for all genes consisted of an initial denaturation step at 95 °C for 30 seconds, followed by 40 cycles as following: denaturation at 95 °C for 10 seconds, annealing at 56–60°C for 30

Primer	Sequence 5'→ 3'	TM	Product size bp
Forward Revers	5′– CGATACAAGGCTGTTAGAGAG –3′ 5′– AAACCAGAAAGACGCTTTCGCTTC–3′	60	220

Table 1: The sequences of primers that was used to investigation colony PCR

Primer	Sequence 5'→ 3'	TM	Product size bp
Forward Revers	Forward: 5′-ATCATTGGCAGAGGACGGAG –3′ Revers: 5′–GCGGAGCAATAAATGAACCA –3′	60	4360

Table 2: Primers used for flanking the targeted region.

Primers Sequence			
primer	Sequence 5'→ 3'	TM	Product size (bp)
Mage–A11	F:CTGACCTGATAGACCCTGAG R: GCATGCATACAGAGGCTTC	60	189
Survivin	F: ACCACCGCATCTCTACATTC R: AGAAGAAACACTGGGCCAAG	60	132
RRM2	F: AGTCAGTTGGTGCCAGATAG R: TCCTTGCCCTGAGAGATTC	57	123
β–actin	F: AGATCATTGCTCCTCCTGAG R: CTAAGTCATAGTCCGCCTAG	56	162
Guid 1 for Mge–A11 gene	Sense: CACCGAAGCGAAAGCGTCTTTCTG Antisense: AAACCAGAAAGACGCTTTCGCTTC		
Guid 2 for Mge–A11 gene	Sense: CACCGTAGGTCAGCAGGCCGAACTG Antisense: AAACCAGTTCGGCCTGCTGACCTAC		

Table 3: The sequences of primers that was used to investigation expression level of Mage–a11, Survivin and RRM2 and guide 1,2 for Mage–a11 gene.

seconds**, and extension at** 72°C for 45 seconds. Relative quantification of gene expression level was calculated using the comparative Ct method and the equation $2^{-\Delta\Delta Ct}$. A housekeeping gene, β–actin, was used for normalization of the target gene expression.

Apoptosis assay

Apoptosis was evaluated by Annexin V–PE/7–AAD Apoptosis Detection Kit (Elab science, United States) 48 hours after transfection. Briefly, cells were Seeded on 6–well plates at a density of 2.5×10^5 cells/well in 2ml of complete culture medium and grown to 70% confluency. Then, categorization and transfection was performed similar to the section "2.3. Cell transfection". Next, cells were harvested by centrifugation at 500×g for 5 minutes (4°C). Afterward, the cells were washed with 400 µl PBS, were resuspended in 400 µl binding buffer and were stained with 5 µl of annexin V–PE and 5 µl of 7AAD for 15 minutes in the dark at room temperature. Then, the cells were gently resuspended in 400 µl of binding buffer, were kept on ice and were analyzed by FACS Calibur flowcytometer (Becton Dickinson Biosciences, USA).

Data Analysis

Data were presented by the mean ± SEM of the three runs per group. The one–way ANOVA test (followed by Tukey's test) was used to analyze the data. SPSS software version 23 was used for the statistical analyses. P values at <0.05 were considered as significant in all assessments.

Results

Confirming the insertion of sgRNAs into the correct position on the vector

The results of colony PCR confirmed that sgRNAs were successfully inserted into the vector (figure 1–B). In addition, the results of Sanger sequencing confirmed that the sgRNAs sat on the correct position of the vector (figure 1–C).

Analyzing the Transfection efficiency in PC3 cell line

The results of fluorescent microscopy (figure 2–A), as well as the results of flow cytometry (figure 2–B), showed that the PC3 cells were efficiently transfected with sgRNA–containing vectors.

Knockout of MAGE–A11 by CRISPR/Cas9

The results of Genomic PCR displayed that MAGE–A11 gene was successfully knocked out in the transfected PC–3 cells. However, the gene was still present in the control and the scramble groups (figure 2–C).

The results of qPCR analyses displayed that the expression level of MAGE–A11 reduced remarkably in

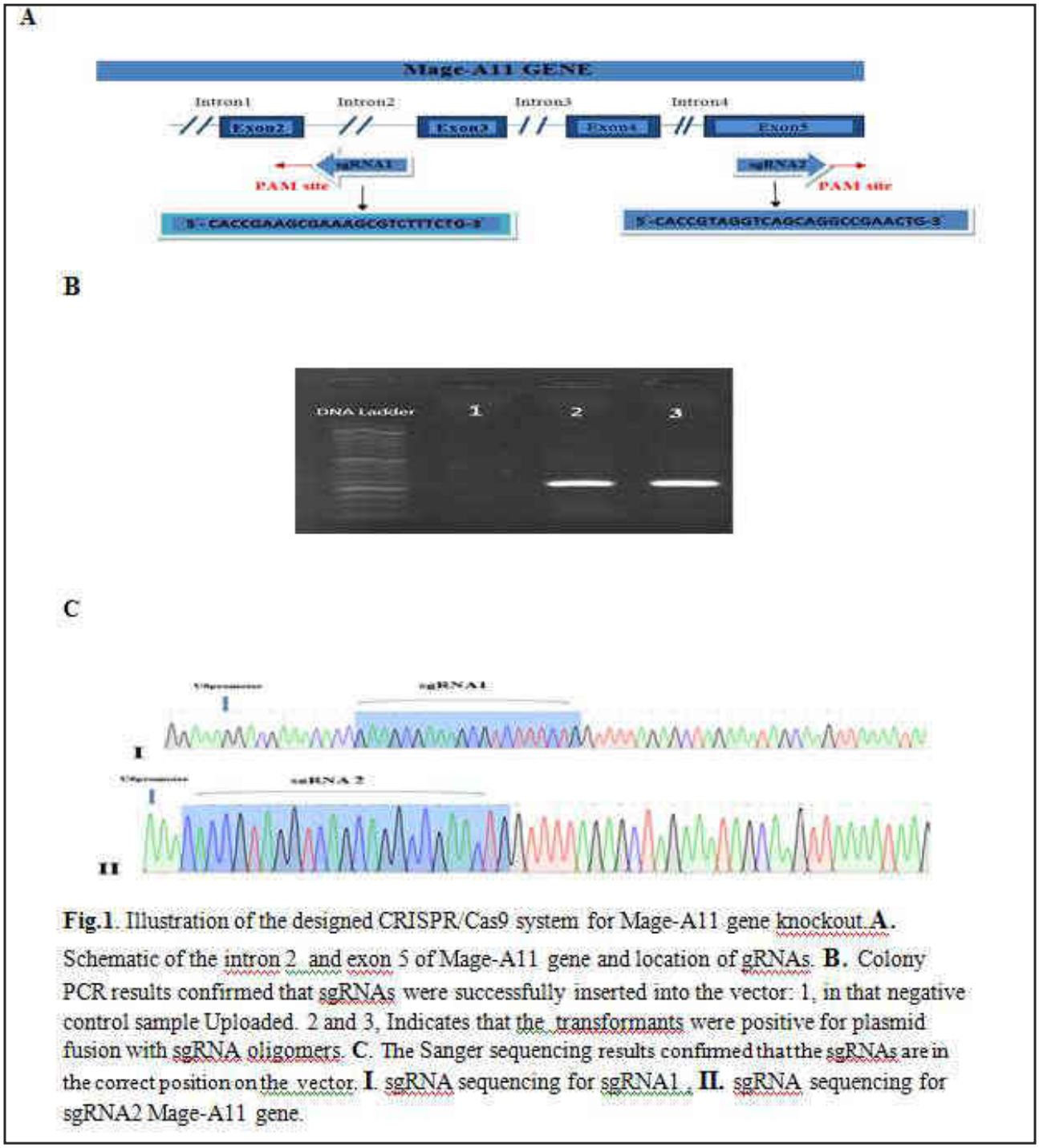


Fig.1. Illustration of the designed CRISPR/Cas9 system for Mage-A11 gene knockout. **A.** Schematic of the intron 2 and exon 5 of Mage-A11 gene and location of gRNAs. **B.** Colony PCR results confirmed that sgRNAs were successfully inserted into the vector: 1, in that negative control sample Uploaded. 2 and 3, Indicates that the transformants were positive for plasmid fusion with sgRNA oligomers. **C.** The Sanger sequencing results confirmed that the sgRNAs are in the correct position on the vector. **I.** sgRNA sequencing for sgRNA1 , **II.** sgRNA sequencing for sgRNA2 Mage-A11 gene.

the treatment group compared to control group ($p<0.001$) .However, no significant difference was observed between control group and scramble group (figure 2–D).

Analyzing the expression levels of survivin and RRM2 genes

The results of qPCR analyses showed that the expression level of survivin and RRM2 genes were decreased significantly compared to control group ($p<0.001$). However, no significant difference was observed between control group and scramble group, regarding the expression levels of survivin and RRM2 genes (figure 3–A and figure 3–B).

Analyzing the levels of the cell proliferation and the apoptosis

The results of cell proliferation assay, 24 hours and 48 hours after the transfection, showed a significant decrease in the proliferation of the cells within the treatment group compared to the control group ($p<0.001$). However, no

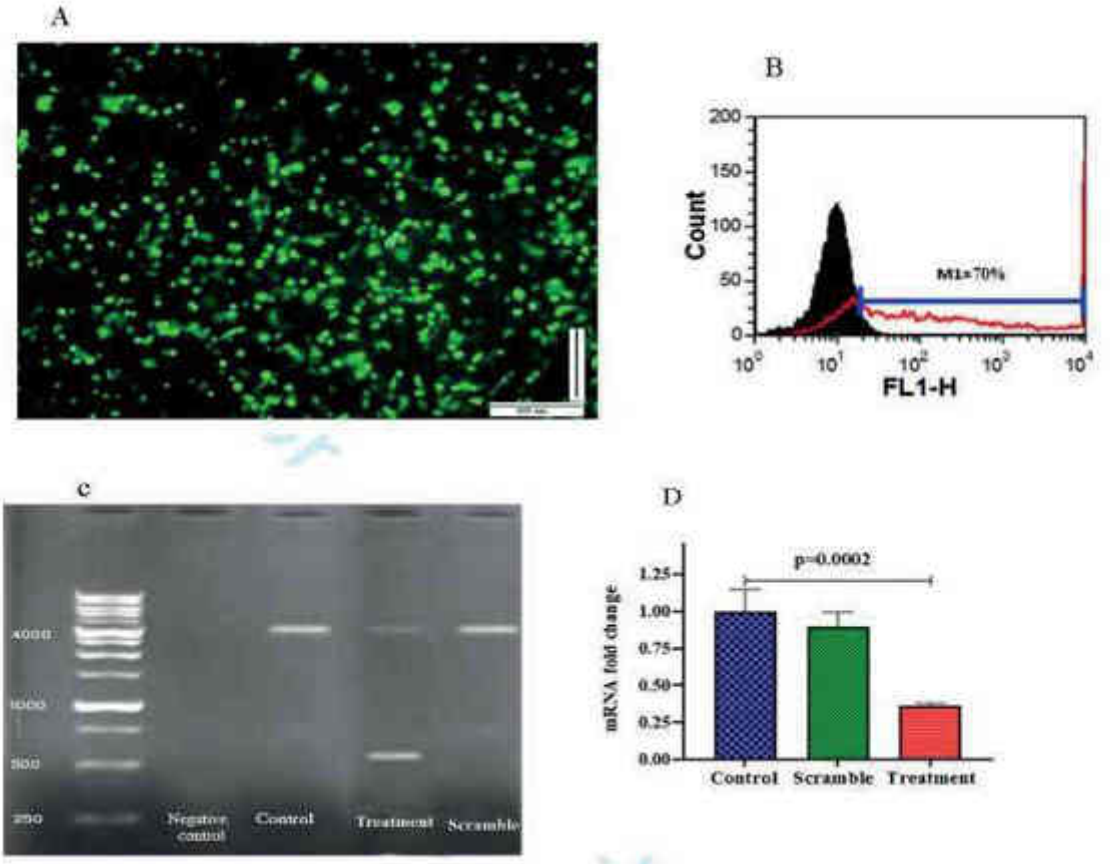


Fig .2. Analyzing the Transfection efficiency in PC3 cell line by PCAG-eCas9-GFP. Pc3 cells were seeded in six-well plates at a density of 5×10^5 cells/well and grown to 70% confluence, the cells were cotransfected by using Lipofectamine 3000. A. Fluorescent microscopy images of pc3 cells transfected by vectors containing sgRNA after 48 hours (scale bars: 200μm). B. The results of the assesment of transfected cells by flow cytometry showed the PC3 cells were effectively transfected with vectors containing sgRNA (more than 70 %). **C.** The Genomic PCR depicting the deletion of MAGE-A11 gene in the treatment group (band at 650 bp) compared to the control and scramble groups (band at 4360 bp). **D.** The expression levels of Mage-A11 gene within the experimental groups. The expression level of MAGE-A11 decreased significantly in the treatment group compared to the scramble and control group (p=0.0002).

significant difference was observed between control group and scramble group, regarding their proliferation. (figure 4–A)

The results of apoptosis assay showed that apoptosis increased significantly in treatment group compared to control group ($p<0.001$). However, no significant difference was observed between control group and scramble group, regarding their apoptosis level (figure 4–B).

Discussion

The results indicated that the disruption of MAGE–A11 result in reduced cell proliferation and increased of apoptosis in PC3 cell line as a prostate cancer–derived cell line. Consistently, Minges et al. (2013) demonstrated that the knockdown of MAGE–11 by a small hairpin RNA in LAPC–4 cell line (a prostate cancer–derived cell line) led to inhibition of cell proliferation and tumor growth via inhibition of AR transcriptional activity[5]. Furthermore, Su et al. (2013) showed that the overexpression of MAGE–A11 could induce retinoblastoma–related protein p107 as a transcriptional activator of E2F1, which in turn could lead to increased proliferation of prostate cancer cells[16].

Recent research results showed that expression levels of survivin and RRM2 genes decreased due to knocking out the MAGE–A11 gene. This finding suggested that survivin and RRM2 are potential downstream key players in signaling of MAGE–A11 and may participate in cell proliferation and tumor progression. In this regard,

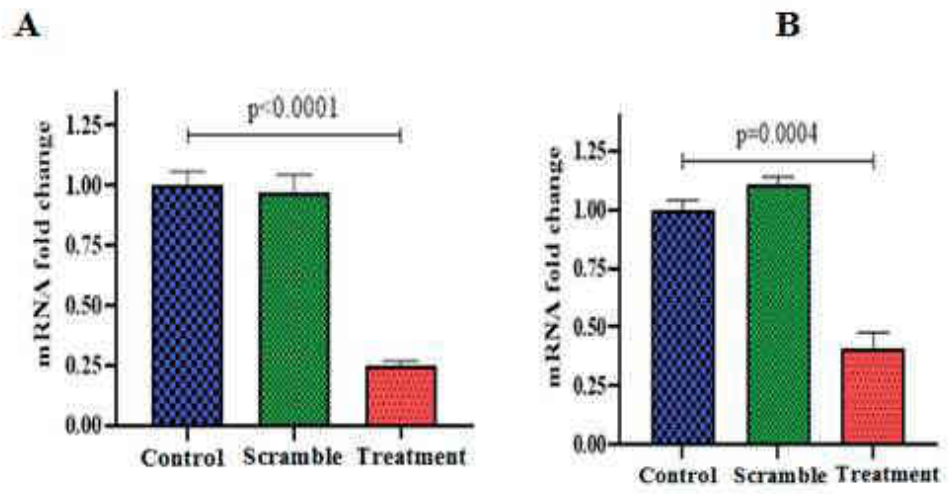


Fig3. Analyzing the expression levels of survivin and RRM2 genes. Effect of knocking out of Mage-A11 gene on expression levels of survivin and RRM2 in PC3 cell line via qRT-PCR 48 h after transfection. **A.** Expression level of survivin gene was decreased significantly in Treatment group compared to scramble and control groups ($p < 0.0001$). **B.** RRM2 expression levels was significantly reduced in Treatment group compared to scramble and control groups ($p=0.0004$).

previous studies showed that RRM2 was frequently upregulated in various cancers including prostate cancer and increased genomic instability, cancer progression and resistance to treatments [23, 26, 27]. RRM2 is essential for DNA production and DNA repair by dNTP production[28]. Huang M.D et.al (2014) showed that RRM2 was overexpressed in advanced prostate cancer and played a key role in the proliferation and cancer invasion[29]. They stated that upregulation of RRM2 increased Cell invasion and the metalloprotease MMP–9 expression, which in turn led to tumor progression in prostate cancer. Additionally, an increase in RRM2 could activate the Ras/Raf pathway, which in turn participated in recurrence of prostate cancer[29]. Furthermore, previous studies showed that overexpression of RRM2 significantly increased proangiogenic vascular endothelial growth factor (VEGF) and induced angiogenesis in prostate cancer[29].

Consistent with the results of Recent research , previous studies frequently indicated which survivin is upregulated in many human cancers including prostate. Indeed, survivin is correlated with inhibition of apoptosis, increased cell proliferation and tumor growth[19]. Survivin is a component of the chromosomal passenger complex (CPC) that regultes the exact separation of chromosomes and cytokinesis during cell proliferation [30]. Moreover, survivin modulates apoptosis via inhibiting caspase–3 and caspase–7 as well as p53. Jianjun Shen and et.al (2014) showed that Knockdown of survivin gene expression by siRNAs in PC–3 cells led to significant reduction in cell proliferation via induction of apoptosis. To our best of knowledge, this research was the first evidence on presence of a functional correlation between MAGE–A11 and survivin.

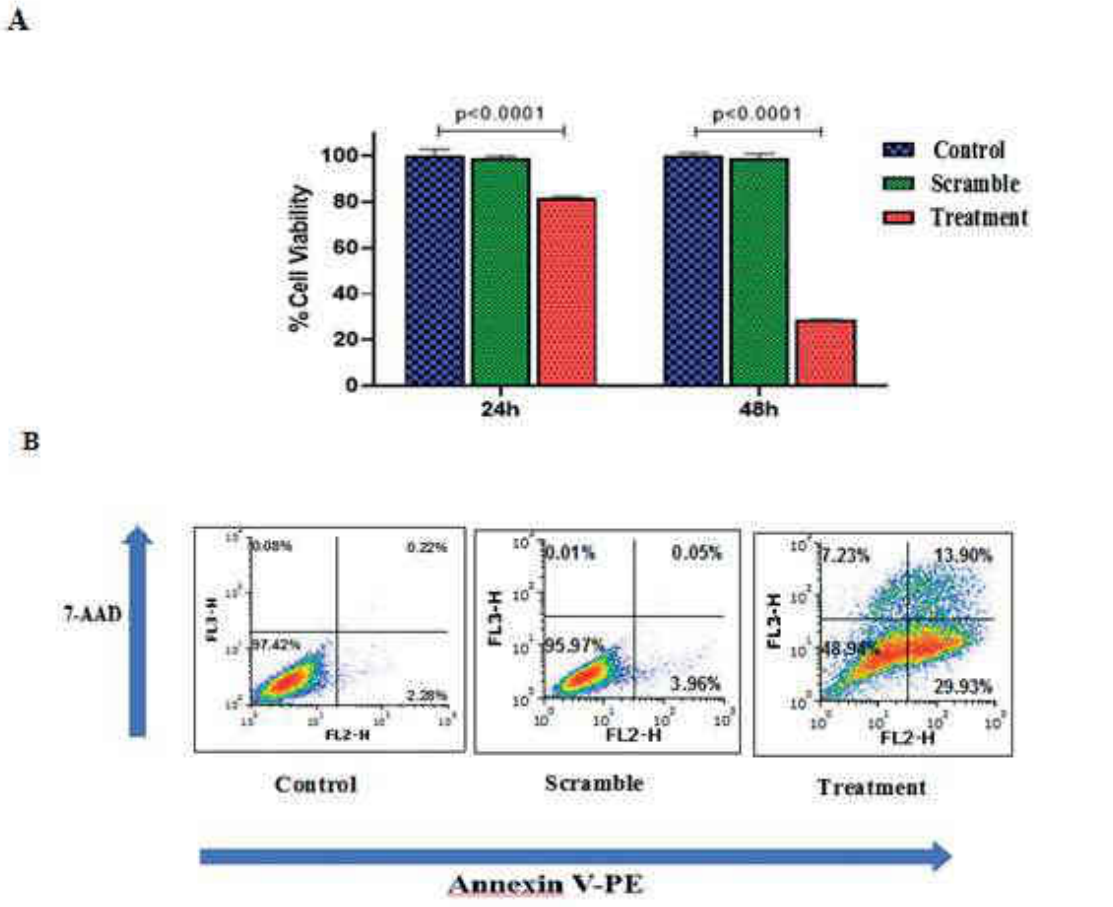
Current study could show a probable link between MAGE–A11 and two other genes (Survivin and RRM2) (figure 5). However, further investigations are needed to prove the actuality of the link and to elucidate their possible precise mechanism of interaction. Additionally, current study indicated that knocking out of MAGE–A11 using CRISPR–Cas9 technique could efficiently disrupt cell proliferation of PC–3 cell line (as a prostate cancer–derived cell line) and induce apoptosis in them. Accordingly, MAGE–A11 may be a promising therapeutic target for treatment of prostate cancer. However, undoubtedly further preclinical and clinical investigations are needed in this regard.

Conclusion

Our results demonstrated that the knocking out MAGE–11 gene by CRISPR/CAS9 technique could inhibit cell proliferation and progresion of tumor and downregulate the survivin and RRM2 genes expression level in PC3 cell line.

Compliance with ethical standards:

Acknowledgements: This work was supported by a grant (MUK.1396/126) from KUMS to AJ. LF is a PhD candidate at KUMS and this work was a part of her thesis.



4.Analyzing the levels of the cell proliferation and the apoptosis.A. The results of cell proliferation assay, 24 hours and 48 hours after the transfection, showed a significant decrease in the proliferation in the treatment group compared to the scramble and control groups ($p<0.0001$).B. The results of apoptosis assay showed that apoptosis increased significantly in treatment group compared to scramble and control groups ($p<0.0002$).

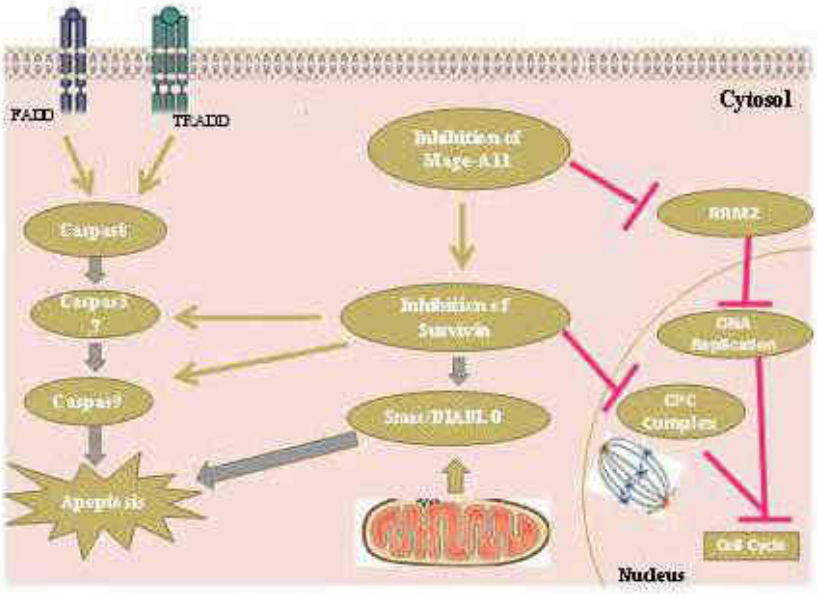


Fig.5. Illustration of the link between MAGE-A11 and two other genes (Survivin and RRM2).

Conflict of interests and disclosure: The authors declare that they have no competing financial interest and nothing to disclose.

Data sharing: All data generated or analyzed in this study are available upon reasonable reques.

Ethical standards:

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

Author contribution: All of these authors contributed to the article. AJ and LF designed research. LF, SHF and FS performed experiments. LF and AJ wrote the manuscript and AJ reviewed it. The authors declare that they have no competing financial interest and nothing to disclose. All authors read and approved the final version.

Consent to Publish:

All authors agree and consent to the publication.

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