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Epigallocatechin-3-gallate Enhances the Efficacy of MicroRNA-34a Mimic and MicroRNA-93 Inhibitor Co-transfection in Prostate Cancer Cell Line

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ABSTRACT

The potential role of microRNAs (miRNA or MIR) as therapeutic molecules has moved them from basic research to the field of cancer therapy. High expression of miR-93 and low expression of miR-34a have previously been indicated in prostate cancer (PC), which is the second leading cause of cancer-related death in men. Androgen receptor (AR) and prostate-specific antigen (PSA) play key roles in the initiation and progression of this cancer. Therefore, this study aimed to investigate the effects of the transfection and co-transfection of miR-34a mimic and miR-93 inhibitor with or without epigallocatechin-3-gallate (EGCG) on prostate cancer cell line and also to evaluate their effects on the expression of AR, PSA.

Human lymph node carcinoma of the prostate (LNCaP) cells were treated with miR-34a mimic or/and miR-93 inhibitor with or without EGCG. Gene or protein expressions were assessed by real-time PCR or western blotting of lysates.

The transfection with miR-34a mimics significantly reduced the mRNA expression of AR (p=0.0016), and PSA (p=0.038) compared to the control. Also, the miR-93 inhibitor led to a decrease in the mRNA expression of AR (p=0.0057) and PSA (p>0.05) compared to the control group. Furthermore, the co-transfection, along with EGCG, caused more decrease in both the AR (p<0.001) and the PSA (p=0.003) expression compared with the co-transfection without EGCG.

Our study indicates that the reduced expression of AR and PSA in PC cells followed by treatment with miR-34a mimic and miR-93 inhibitor and their combination with EGCG as a natural substance may be a promising therapeutic way for controlling the growth of these malignant cells.

Keywords: Androgen receptor; MicroRNAs; Prostate cancer; Prostate-specific antigen

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INTRODUCTION

Prostate cancer (PC) is one of the most common cancers in men.[1] Androgen receptor (AR) is a member of the nuclear receptor superfamily, which plays key functions in the initiation and progression of PC.[2] In the absence of an androgenic ligand, AR exists primarily in the cytoplasm, where it exists as a complex with heat shock proteins. Conformational changes occur in the AR following ligand binding, allowing AR homodimer formation and its translocation into the nucleus as a transcription factor. AR has a crucial role in the transcription of prostate-specific antigen (PSA), which is contributed to the growth, development, and survival of PC.[3,4]

Given prostate tumors are initially dependent on androgens for growth and survival. Androgen deprivation is the mainstay treatment for advanced and metastatic PC.[5,6] However, AR activation could occur in the absence of an androgenic ligand and cause a continuous transcription leading to an increase in the proliferation of cancer cells and a progression in the clinical complications.[6,7] Therefore, developing an approach for reducing the expression of AR or for preventing its transcriptional activity could be regarded as novel goals along with current treatments for PC.

Epigenetic processes connect genes and the environment, mediated by DNA methylation, histone modification, and microRNA (miRNA, miR) alterations.[8] MiRNAs are a class of small, non-coding, and endogenous RNAs regulating the gene expression at the post-transcriptional level through complementary binding to the 3′-untranslated regions (3′-UTRs) of target genes, leading to the degradation of target mRNA or repression of mRNA translation.[9-11]These small RNAs can act as both a tumor suppressor gene and an oncogene depending on their cognate targets.[12, 13] Deregulation of miRNA genes in cancers is related to mutations, epigenetic changes, and alterations in the processing of miRNAs.[14] The importance of miRNA networks in the management of cellular dysregulations becomes more apparent when noting that some miRNAs such as miR-16, miR-155 are under investigation in human trials for the treatment of lung cancer and T cell lymphoma respectively.[15] It was shown that miR-34a contributes to the expression of target proteins involved in the cell cycle, apoptosis, and differentiation, and antagonizes processes that are essential for the viability of cancer cells and metastasis, and chemoresistance. MiR-93 was found to influence the proliferation and metastases of many cancers. The upregulation of miR-93 and the downregulation of miR-34a has been associated with PC.[16-20]

The anticarcinogenic effects of green tea as one of the most widely consumed beverages have been attributed to its polyphenols, particularly epigallocatechin-3-gallate (EGCG), which is the most abundant and the most biologically effective polyphenol.[21,22] EGCG effective against the initiation, progression, and invasion of different cancers such as PC.[22,23] EGCG can contribute to modulating the gene expression through affecting epigenetic processes such as DNA methylation and/or histone modification. This important polyphenol can change DNA methylation patterns in cancer cells by directly and indirectly inhibiting DNA methyltransferases (DNMTs).[24,25] It was reported that cancer cells treated with EGCG indicated decreased DNA methylation and increased transcription of tumor suppressor genes.

This study, therefore, aimed to investigate the effects of the transfection and co-transfection of miR-34a mimic and miR-93 inhibitor with or without epigallocatechin-3-gallate (EGCG) on prostate cancer cell line and also evaluate their effects on the expression of AR, PSA in these cells.

MATERIALS AND METHODS

Materials

This study was approved by Shahid Beheshti University of Medical Sciences (Ethics committee approval code: 375)

Lymph node carcinoma of the prostate (LNCaP) cell line was obtained from the Pasture Institute of Iran (Tehran, Iran). Roswell Park Memorial Institute Medium (RPMI) 1640, OPTI-MEM medium, and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA). EGCG, bovine serum albumin (BSA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT powder) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000, RevertAid reverse transcriptase, and RNase free DNase were provided from Thermo Fisher Scientific (USA). All miRNA sequences, real-time PCR primers, and Accuzol total RNA extraction reagents were obtained from Bioneer (Seol, South Korea). SYBR Green Premix Ex Taq master mix and cDNA Synthesis Kit

were gained from Takara (Dalian, China). Polyvinylidene Difluoride (PVDF) membrane was obtained from Bio-Rad Laboratories (CA, USA). Anti-AR mouse IgG1 monoclonal antibody was purchased from Abnova (Taipei, Taiwan), anti-β-actin, goat anti-rabbit IgG H & L-HRP, and goat anti-mouse IgG-HRP were provided from Abcam (Cambridge, UK). Protein extraction RIPA buffer was provided from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Treatment of Cells with EGCG

LNCaP cells were cultured in RPMI 1640 supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. EGCG was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution, and aliquots of stock solution were stored in the dark at −20°C. Treatment of LNCaP cells with EGCG was carried out for 48 h at 37°C. Following the treatments, cells were used to determine cell viability, morphological features, and expression of specific miRNAs, AR, and PSA.

Determination of Cell Viability; Using MTT Assay

LNCaP cells were seeded in 96 well culture plates (5×10^3 cells/well) in 200 µl RPMI 1640 containing 10% FBS for 24 h. The cells were then treated with EGCG (20, 40, and 60 µg/mL) or were transfected with miR-34a mimic (50 pM), miR-93 inhibitor (100 pM), either alone or in combination for 48 h. After the treatments, the medium was discarded and then incubated with 0.2 mg/mL MTT solution for 4 h. Then, 200 µL DMSO was added to each well to dissolve the formazan crystals. The optical densities were measured at 570 nm with background subtraction at 630 nm. The viability of the non-treatment cells (control) was defined as 100%, and the viability of cells from all other groups was calculated separately from that of the control group. The relative cell viability was reported as the percentage of absorbance compared to the control group. The growth inhibition was determined based on the following formula: The Growth inhibition (%)=[(the absorbance of the control group–the absorbance of the experimental group)/the absorbance of the control group]×100.

Transfection of MiRNAs

After 24 hours of incubation at 37°C, 5% CO_2, miR negative control, miR-34a mimic (50 pM), miR-93 inhibitor (100 pM), miR-93 inhibitor-NC (100 pM) were transfected using lipofectamine 2000 according to the manufacturer's instruction. In brief, lipofectamine 2000 was diluted in OPTI-MEM and was added to the diluted miRNAs. After incubation at room temperature for 20 min, the mixture was added to the cell suspension and then incubated for a further 48 h.

RNA Extraction and cDNA Synthesis

The treated cells were collected and washed with PBS. The cell pellet was used in total RNA extraction using Accuzol reagent, following the manufacturer's instructions. The quality and purity of the RNA were assessed using a Picodrop instrument (PicoDrop, France). According to the manufacturer's instruction, the extracted total RNA was proceeded to DNA removal using an RNase-free DNAase kit.

The first strand complementary DNA (cDNA) for mRNA evaluations was generated from total RNA using random hexamer primers and RevertAid reverse transcriptase. In miRNA experiments, specific stem-loop reverse transcription primers were exploited instead of random hexamer primers for specific cDNA synthesis.

Real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) amplification was performed using a Bioneer thermal cycler system with SYBR green as a fluorescent reporter. MiR-34a, miR-93, and gene-specific primer sets for human PSA, AR, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes are represented in Table 1. GAPDH was used as an internal control to normalize the relative expressions. For miRNAs analysis, the U6 small nuclear RNA was used as the internal control. The mean threshold cycle number (Ct) of the triplicate reactions was determined. The levels of specific gene expression were normalized to GAPDH or U6 levels using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (calibrator) and ΔCt is the Ct of the housekeeping gene subtracted from the Ct of the target genes. Melting curves were also determined for the identification of primer-specific amplicons.

Western Blot Analysis

For western blotting, the cells were lysed using RIPA buffer. The protein concentrations were measured via Bradford assay. Proteins were run in an

SDS-PAGE gel and then transferred to a 0.2 µm immune-blot PVDF membrane. The membranes were blocked with 5% (w/v) BSA in 0.1% Tween 20 for 1h. The membranes were incubated with anti-AR antibody and anti-β-actin loading control for 1 h at room temperature. Subsequently, membranes were washed thrice with TBST and incubated with goat anti-rabbit IgG, H&L-HRP, and goat anti-mouse IgG-HRP secondary antibodies for 1h. Western blots were then incubated with enhanced chemiluminescence (ECL) substrate for 12 min. The protein expression was normalized to the expression of β-actin as a house-keeping internal control. Densitometry of protein bands was performed using the Image J software (Version 1.44, USA). The area under the curve of each test band was divided by the area under the curve of its corresponding β-actin band.

Statistical Analysis

Data are presented as mean±SD. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *p* values of less than 0.05 are considered statistically significant. All the statistical analyses were performed; using the software GraphPad Prism version 6.0 (GraphPad Software Inc, La Jolla, CA, USA).

RESULTS

EGCG Suppresses the Proliferation of LNCaP Cells

EGCG suppressed the growth of LNCaP cells in a dose-dependent manner (Figure 1A). The percentage of growth inhibition at 20, 40, and 60 µg/mL of EGCG in 48h were 19±5.4%, 44.7±2.4%, and 76.4±3.5%, respectively (Figure 1A). As the dose of EGCG increased, the cells represent an altered morphology including, fragmented nuclei and wrinkled membrane (Figure 1B). As shown in Figure 2, the half-maximal inhibitory concentration (IC_{50}) of EGCG was obtained to be 42.2 µg/mL.

Table 1. The sequences of the primers used in gene expression analysis

Primer	Sequence (5′- 3′)
miR-34 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAACC
miR-93 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCT
U6 RT primer	GAATTTGCGTGTCATCCTTG
miR-34a	
F	GGGATGGCAGTGTCTTAGC
R	GTGCAGGGTCCGAGGT
miR-93	
F	GGACAAAGTGCTGTTCGTGC
R	GTGCAGGGTCCGAGGT
AR	
F	CTTCCCTCCCTATCTAACCCTC
R	TCTAAACTTCCCGTGGCATAA
PSA	
F	AGGTGTGCTGACTATGTGGTGAC
R	GGTTGAGGTTCCAGGTGCTT
GAPDH	
F	CATGAGAAGTATGACAACAGCCT
R	AGTCCTTCCACGATACCAAAGT
U6	
F	GCTTCGGCAGCACATATACTAAAAT
R	CGCTTCACGAATTTGCGTGTCAT

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; AR: androgen receptor; PSA: prostate-specific antigen

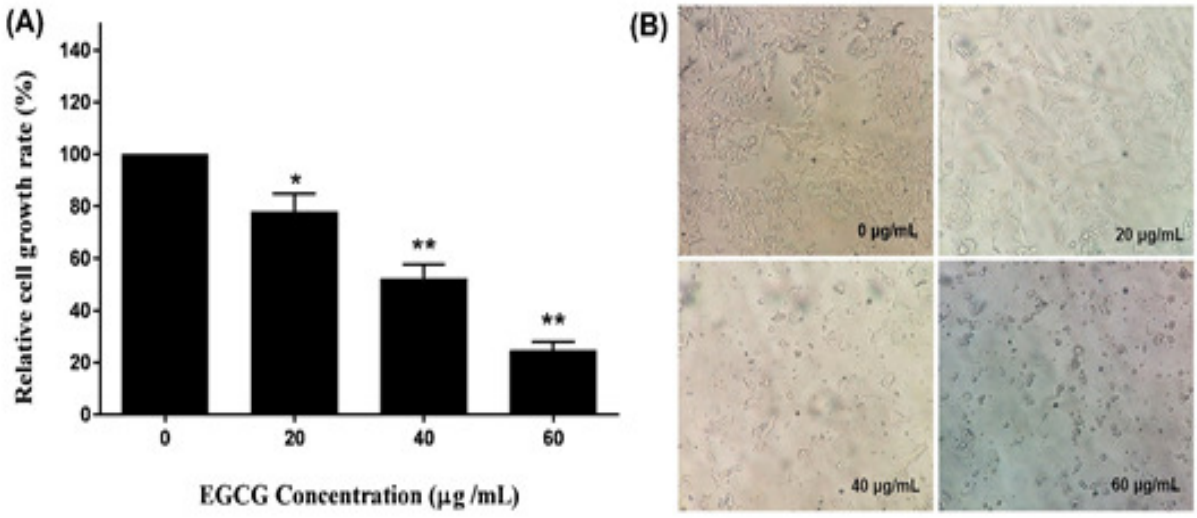

Figure 1. (A) The Effects of epigallocatechin-3-gallate (EGCG) on the growth of LNCaP cells. EGCG suppressed the growth of these cells in a dose-dependent manner. (B) Morphological changes after 48 h of treatment with EGCG. Results are expressed as means ± SD of three independent experiments. *p<0.05, **p<0.01

EGCG Inhibits the Expression of AR and PSA in LNCaP Cells

As shown in Figure 3, the relative expression of AR (Figure 3A) and PSA (Figure 3B) decreased at the concentration of 20 µg/mL EGCG; compared to the control group. However, there was no significant difference between the groups.

Effects of MiR-34a Mimic and MiR-93 Inhibitor on the Proliferation of LNCaP Cells

The MTT assay revealed that miR-34a mimic and miR-93 inhibitor could inhibit the growth of LNCaP cells (Figure 4). The cell viability is significantly inhibited by miR-34a mimic and miR-93 inhibitor. Furthermore, co-transfection of miR-34a mimic and miR-93 inhibitor, and their co-transfection along with EGCG, significantly reduced the proliferative capacity of LNCaP cells. As shown in Figure 5, the miR-34a mimic + miR-93 inhibitor (p<0.01) and miR-34a mimic + miR-93 inhibitor + EGCG (20 µg/mL) (p<0.001) significantly inhibited LNCaP growth rate. Accordingly, the co-transfection of the miR-34a mimics and miR-93 inhibitor together with EGCG (20 µg/mL) had the most potent inhibitory effect on cell proliferation (Figure 4).

Effects of MiR34a Mimic and MiR-93 Inhibitor on the mRNA Expression of AR and PSA

To investigate the potential effects of miR-34a and miR-93 on the AR and PSA expression, LNCaP cells were transfected with miR-34a mimic (50 pM) and miR-93 inhibitor (100 pM) for 48 h (Figure 5). The results indicated that the transfection with miR-34a mimics significantly reduced the mRNA expression of AR (p=0.0016) and PSA (p=0.038) compared to control. Moreover, as shown in Figure 5, miR-93 inhibitor led to a decrease in the mRNA expression of AR (p=0.0057) and PSA (p=0.1557) compared to the control group.

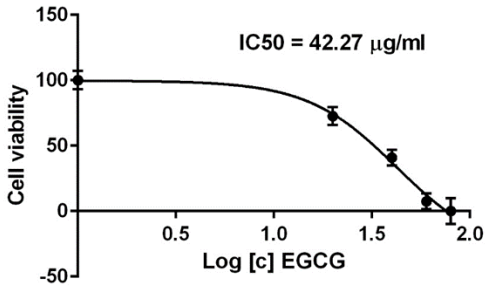

Figure 2. IC50 graph of LNCaP cells treated with epigallocatechin-3-gallate (EGCG)

Effects of Co-transfection of MiR-34a Mimic and miR-93 Inhibitor Alone or Along with or without EGCG on the Expression of AR and PSA

For co-transfection analyses, 5×10^4 cells/well were seeded in 24-well plates for 24 h, and then the culture medium was removed and replaced with a fresh medium. Then the treatments were carried out at the 20 µg/mL concentration of EGCG along with co-transfection. After 48h, the AR and PSA expressions were analyzed. As indicated in Figure 6, the co-transfection of miR34a mimic and miR-93 inhibitor led to a decrease in the expression of AR (p=0.0019) and PSA (p>0.05) compared with the control group. Also, the co-transfection, along with 20 µg/mL EGCG, significantly reduced the mRNA expression of AR (p<0.001) and PSA (p=0.003) in comparison to control. The results demonstrated that the co-transfection along with 20 µg/mL EGCG caused more decrease on both the AR (p<0.001) and the PSA (p=0.003) expression compared with the co-transfection without EGCG (Figure 6).

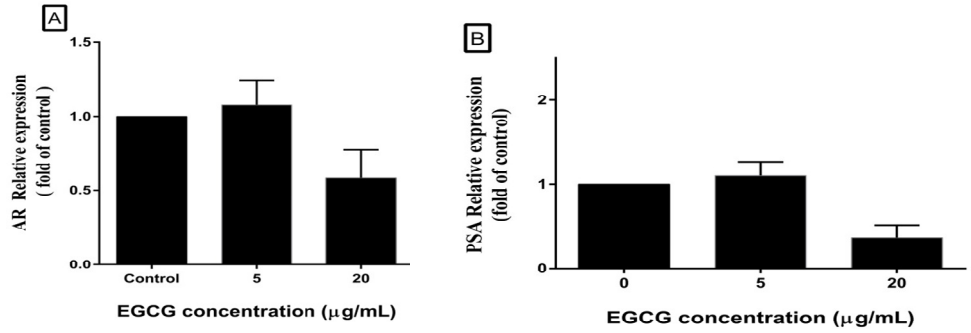

Figure 3. The Effects of epigallocatechin-3-gallate (EGCG) on the mRNA expression of (A) androgen receptor (AR) and (B) prostate-specific antigen (PSA) in LNCaP cells. Results are expressed as means±SD of three independent experiments with no significant differences

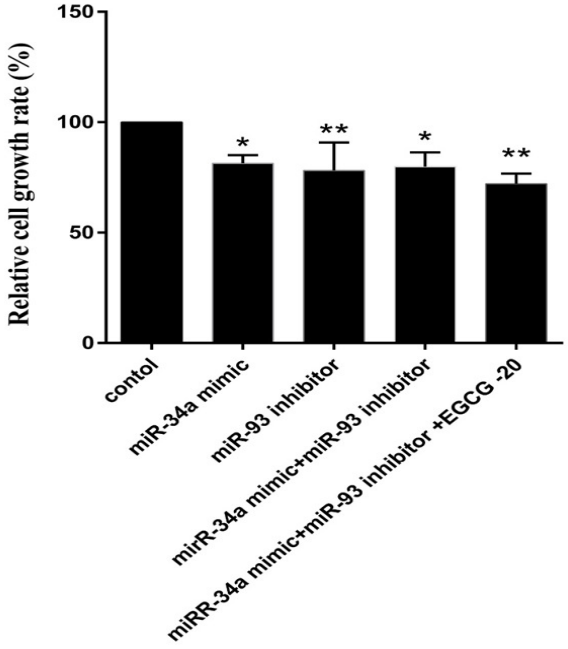

Figure 4. The effects of miR-34a mimic and miR-93 inhibitor with or without epigallocatechin-3-gallate (EGCG) on the proliferation of LNCaP cells compared to the control group. *p<0.05, **p<0.01, ***p<0.001

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Western Blot Analysis of AR Protein in LNCaP Cells

Our western blot analysis exhibited that AR protein expression underwent a significant decrease in 20 µg/mL EGCG treated co-transfected cells (Figure 7). Also, a reduction in AR protein expression was observed in the miR-34a mimic transfection and the miR-93 inhibitor.

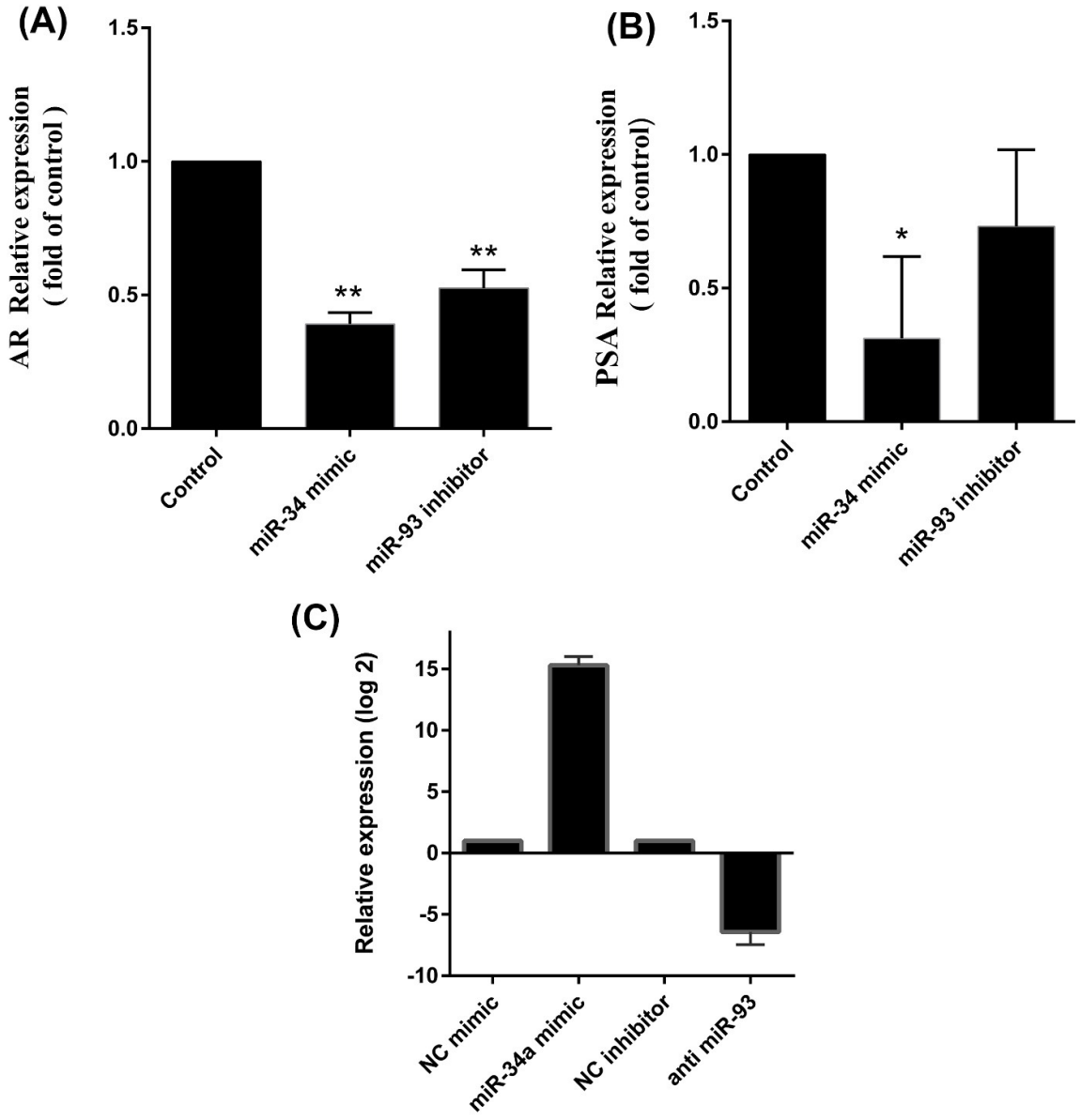


Figure 5. The effects of miR34a mimic and miR-93 inhibitor on the mRNA expression of (A) androgen receptor (AR) and (B) prostate-specific antigen (PSA) in LNCaP cells. (C) The comparative levels of miR34a and miR-93 following 48 h transfection with miR-34a mimic and miR-93 inhibitor in LNCaP cells compared to the control group. Results are expressed as means±SD of three independent experiments. **p<0.01, *p<0.05

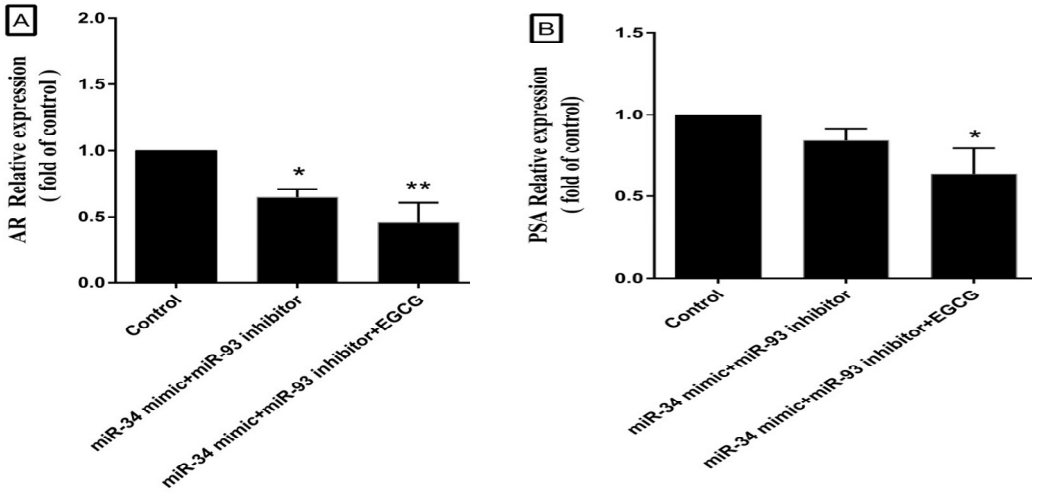
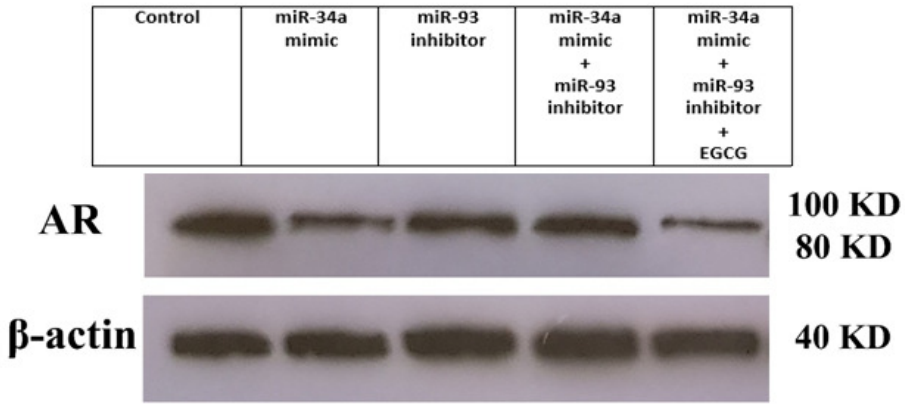


Figure 6. The effects of co-transfection of miR34a mimic and miR-93 inhibitor alone or along with 20 µg/mL epigallocatechin-3-gallate (EGCG) on the expression of (A) androgen receptor (AR) and (B) prostate-specific antigen compared to control group. Results are expressed as means±SD of three independent experiments. **p<0.001, *p<0.05

(A)



(B)

	Control	miR-34a mimic	miR-93 inhibitor	miR-34a mimic + miR-93 inhibitor	miR-34a mimic + miR-93 inhibitor + EGCG
AR	5226	2095	3768	4016	1260
β-actin	5667	5689	6189	6670	5268
Relative Intensity	0.922	0.368	0.608	0.602	0.239

Figure 7. (A) Western blot analysis of androgen receptor (AR) protein in LNCaP cells. (B) Densitometry results of the protein bands

DISCUSSION

EGCG has been demonstrated to possess intriguing effects on cancer prevention and therapy in various cancers, including prostate cancer.[22,23] However, molecular mechanisms and direct targets of EGCG, particularly its interactions with miRNAs, are not yet well clear. According to our findings, EGCG inhibited the proliferation of LNCaP human prostate cancer cells in a dose-dependent manner. Moreover, miR-93 inhibitor and miR-34a mimic inhibited the growth of these cells and, more importantly, EGCG (20 µg/mL) enhanced their inhibitory effects. Studies have shown that the effects of EGCG on cancer cell growth may exert through various mechanisms including regulating the expression of miRNAs.[26,27]

Mir-34 is one of the tumor suppressor miRNAs that its expression decreases in several cancers including PC.[28,29] This miRNA targets various genes related to cell cycle, oncogenesis, and metastasis such as Myc, CDK4, CDK6, CREB, and Met.[29-31] MiR-93 as a member of the pro-oncogenic miR106b-25 cluster is strongly expressed in PC and may play a role in regulating cellular proliferation, survival, migration, apoptosis, cell cycle, and angiogenesis.[32-34] Based on our findings, the enhanced effects of miR-34a mimic and miR-93 inhibitor on prostate cancer cells occur in the presence of EGCG, which may be one of the molecular mechanisms that this polyphenol decreases the growth of these cells. The results presented herein are supported by Chakrabarti et al[35] reported that EGCG decreases the expression of oncogenic miRNAs, including miR-92, miR-93, and miR-106b and increases the expression of tumor-suppressor miRNAs, including miR-7-1, miR-34a, and miR-99a in neuroblastoma SK-N-BE2 and IMR-32 cells. Also, Tsang et al.[36] Using miRNA microarray analysis showed that the expressions of 13 miRNAs including miR-93 were upregulated while EGCG downregulated the expressions of 48 miRNAs including miR-34b in hepatocellular carcinoma HepG2 cells. Furthermore, according to the findings by Toden et al,[37] EGCG upregulated self-renewal suppressive-miRNAs, including miR-34a, miR-145, and miR-200c, which are some of the key pathways targeted in 5-fluorouracil-resistant colorectal cancer cells.

According to studies, the transcription factors c-Myc, Elk1, FoxO3, Raf, and p53 contribute to miR-34a expression.[37-39] The expression of miR-34 can be regulated by c-Myc via the c-Myc/Arf/Hdm2/p53/miR-34 axes.[39] On the other hand, it has been shown that EGCG decreases the expression of Raf and Myc, which are involved in the transcription of miR-34 (EGCG MYC). Therefore, it should be noted that the effects of EGCG on miR-34a may exert through these transcription factors.

Our findings showed that miR-34a mimic and miR-93 inhibitor decreased AR and PSA expression. As expected, the co-transfection of miR34a mimic and miR-93 inhibitor led to a decrease in the expression of AR and PSA. Furthermore, the co-transfection, along with 20 µg/mL EGCG, caused more decrease in the expression of these proteins. To our knowledge, this is the first report on the co-transfection of miR-34a mimic and miR-93 inhibitor in LNCaP cells and the effects of EGCG along with the co-transfection on AR and PSA in these human prostate cancer cells. AR is a miR-34a target gene that the overexpression of this miRNA inhibits its expression.[40] AR plays an essential role in the onset and progression of PC. The receptor acts as a transcription factor after entrance into the nucleus and binding to its regulatory region, contributes to the transcription of various genes, including PSA.[2,3,41] It should also be noted that PSA may play a role in regulating the mRNA and protein levels of AR and also may contribute to AR signaling under conditions of enhancing PSA concentrations in prostate cancer cells.[42] Therefore, the present findings on the decreasing effects of the co-transfection with EGCG on AR and PSA can be important in the treatment of prostate cancer. Notably, this combination therapy may be considered when androgen ablation therapy leads to treatment resistance.

Noteworthy, miR-34a and miR-34b/c were inactivated by promoter CpG methylation in various cancers.[43]The expression of miR-93 can also be influenced by DNA methylation.[44,45] MiR-93 found to be in the status of hypomethylation and up-regulation in hepatocellular carcinoma.[46] In this context, the effects of EGCG on DNA methylation can be considered. This polyphenol can change gene expression through epigenetic modifications such as a reduction in DNA methylation.[47-49] It appears that reduced DNA methylation by EGCG may influence upregulating miR-34a and, conversely, downregulating miR-93. Therefore, the increased efficacy of miR-34a

mimic and miR-93 inhibitor by EGCG on prostate cancer cells and as well as on the expression of AR and PSA found in the present study may be related to the effects of EGCG on DNA methylation, which needs further research.

The co-transfection of miR-34a mimic and miR-93 inhibitor and EGCG may enhance the beneficial effects of conventional cancer therapies. This may be a potentially fruitful focus of anticancer research studies in the future. It should also be mentioned that the complicated network of feedback between miRNAs and epigenetic pathways appears to form a regulatory epigenetics/miRNA circle. When this regulatory circuit is disrupted, normal physiological functions are intervening with, contributing to various disease processes.[50]

Several miRNAs might regulate miRNA targets multiple genes or a distinct gene. It should be noted that numerous studies have focused narrowly on the specific effect of a given miRNA on a particular mRNA, defined by bioinformatics prediction algorithms, rather than exploring the 'bigger picture' of gene expression regulation as part of an extended network.[51] It seems that the regulation of miRNA interactions in cancer is considerably complicated.[52] According to recent studies, miRNAs can bind to and control other non-coding RNAs, including miRNAs. This process is known as a miRNA: miRNA interaction. The overall result of this interaction can be regulating a miRNA's abundance, with subsequent effects on mRNA regulation.[53] Therefore, attention to these interactions can be important in miRNA-mediated therapies.

In conclusion, our study supports the fact that the use of miRNAs along with EGCG as a natural substance for prostate cancer therapy may provide a potentially useful way to increase the effectiveness of treatment. Our study indicates that the reduced expression of AR and PSA in PC cells followed by treatment with miR-34a mimic and miR-93 inhibitor and their combination with EGCG may be a promising therapeutic way for controlling the growth of these malignant cells. These data also support the importance of miRNAs in mediating the anticancer effects of EGCG.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

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