miR-377 inhibits the tumorous behavior of prostate cancer cells by targeting MYC

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Abstract

Aims

The MYC gene is one of the regulatory and proto-oncogenic genes that is overexpressed in most prostate cancers. Studies have shown that abnormal expression of microRNAs is involved in the onset and development of many different types of human cancer, including prostate cancer.

Materials and methods

In this study, we first evaluated targeting the effect of miR-377 on MYC by luciferase assay. Real-time PCR was used to figure out whether miR-377 could decrease the target gene mRNAs in transfected PCa cell lines (PC3 and DU145). The effects of miR-377 on apoptosis cells, proliferation, cell cycle, and wound healing were analyzed.

Results

We showed that miR-377 targets MYC mRNA by luciferase reporter assay. A significant reduction in MYC mRNA level was detected, following miR-377 transfection in PC3 and DU145 cell lines. The higher levels of miR-377 in PCa cell lines induced apoptosis, reduced proliferation, and migration, and stopped the cell cycle.

Conclusion

All these data reveal that miR-377 functions as a tumor suppressor in PCa and may serve as a potential therapeutic target for the treatment of this cancer.

Introduction

With an estimated 1,600,000 cases and 366,000 deaths worldwide, prostate cancer (PCa) is males’ third malignancy and the fifth most common cause of cancer mortality [1, 2]. The main causes of mortality continue to be metastasis and recurrence of disease, as well as the emergence of hormone-resistant diseases. Therefore, research into the molecular pathways behind PCa progression is crucial and might lead to the development of a novel strategy for the disease's focused treatment [3].

Non-coding RNAs (ncRNAs) called micro-RNAs (miRNAs) attach to the 3' untranslated regions of target genes to regulate gene expression post-transcriptionally [4]. Numerous studies suggested that miRNAs were involved in the growth of tumors and may be utilized to detect human malignancies early [5, 6]. miR-21 levels have been reported to be increased in PCa patients and to be related to a bad prognosis [7]. Another study also shows that miR-409-3p/-5p encourages the development of tumors, the change of
epithelial cells into mesenchymal cells, and the metastasis of human prostate cancer to the bones [8].

miR-377 has generated interest because it suppresses the growth of various cancers [9, 10]. Additionally, it has been demonstrated in NSCLC samples miRNA-377 has down expression and its transfection, suppresses tumor growth by negatively regulating genes involved in the ErbB signaling pathway [11].

Also, in pancreatic cancer samples, miR-377 showed down expression and transfection can suppress cell growth and promotes apoptosis [12]. A Study on prostate cancer cells showed that the expression of 14q32.31 miRNAs such as miR-377 is downregulated in human prostate cancer. Transfection of miR-377 affects malignant cell behaviors, including proliferation, apoptosis, migration, and invasion by targeting FZD4 [13].

The MYC oncogene, which is found on chromosome 8q24, encodes the important transcription factor c-myc, which is essential for controlling metabolism, cell proliferation, and apoptosis. An essential development in the precancerous stage (i.e., PIN) of prostate cancer is the amplification of the MYC oncogene [14]. Many studies have shown that MYC is overexpressed in many cancer, such as prostate cancer [15].

It has been established that MYC mRNA is markedly overexpressed in prostate cancer tumor foci, and this is associated with greater severity of the disease. [16]. In the majority of instances of advanced and metastatic castrate-resistant PC (mCRPC), MYC protein abundance is also raised [17].

Here, we discovered that miR-377 strongly inhibits proliferation, cell cycle, and migration and induces apoptosis in prostate cancer cell lines. The mechanism for this was that miR-377 specifically targeted MYC. Our research indicates that miR-377 could be a useful treatment target for PCa.

Materials And Methods

miRNA selection

Bioinformatics research was done to accept that miR-377 could target the 3'UTR of the MYC gene by using the tools miRBase, TargetScan, MiRanda, and miRWalk.

Cell culture

The human prostate cancer cell lines (PC3 and DU145) were obtained from the National Cell bank of Iran (Pasteur Institute Iran (Tehran, Iran)). PC3 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, (Life Technology, 11875119) while DU145 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium, (Life Technology, 12100046), supplemented with 10% fetal bovine serum,(Life Technology, 1600044) and antibiotics (100 units/mL of penicillin and 100 µg/mL of streptomycin) (Sigma Aldrich, St. Louis, MO, USA) and maintained in a cell incubator with 5% CO2 at 37°C.

microRNA transfections
Pre-miRNA precursors, pre-miR-377, and control pre-miR precursors (scrambled) were obtained from (Life Technology). A 24-well plate was seeded by 0.8 ×106 cells one day before transfection. Using Lipofectamine 2000 (Life Technology, 11668019), according to the manufacturer's instructions, cells were transfected with 100 nM of synthetic miR-377 or scrambled oligonucleotide when their confluence reached 80%. The transfection medium was changed to a new medium containing 10% FBS after 6 hours. Cells from transfected lines were collected 24, 48, and 72 hours after transfection.

**Luciferase reporter assay**

The 3'-UTR of MYC (NM_002467.6) was amplified for luciferase reporter studies using the primer pairs CCGCTCGAGAACTTGAACAGCTACGGAAC(forward) and ATAAGAATGCGGCCGCAGTCAGAGTCAAAGAAAGTAAT(reverse) -Endonuclease restriction sites are indicated by bold sequences). With 100 ng of genomic DNA, 10 pmol of each primer, 2 µM MgCl2, 200 µM dNTP, and 1.5 units of Pfu polymerase (Fermentas, EP050), PCR was carried out in a total volume of 25 µL under the following conditions: 95°C for 5 min; 35 cycles of 95°C for 20 s, 51.5°C for 40 s, 72°C for 2 min and 45 s; and final extension Target gene MYC's 3'-UTR and a unique scrambled sequence (AAGCTTCATAAGGCGCATAGC) were cloned into the psiCHECK-2TM Vector (Promega, C8021) just downstream of Renilla luciferase's stop codon. Dual-Glo Luciferase Assay System was used to conduct luciferase experiments. One day before transfection, 4–5 ×10⁴ PC3, and DU145 cells were grown in each well of a 48-well plate to conduct this test. Following the manufacturer's instructions, the cells were transfected using Lipofectamine 2000 reagent (Life Technology, 11668019) with 400 ng of psiCHECKTM-2 Vector containing, MYC 3'-UTR, and 100 nM of each precursor miRNA (377 or scrambled miRNAs). Renilla luciferase activity in the multi-well plate luminometer was standardized to firefly luciferase activity. A dual luciferase reporter assay kit was used to measure luciferase activities 24 hours after transfection (Promega, USA).

**RNA isolation, cDNA synthesis, and quantitative real-time PCR**

Using the miRNeasy Mini kit, total RNA was extracted from PC3 and DU145 after transfection with each precursor miR (miR-377 and miR-scrambled). Utilizing UV spectrophotometric measure total RNA's quantity and quality of total RNA was determined. The cDNA of the mRNA was generated using the cDNA synthesis kit (Fermentas, MA, USA). Using real-time PCR Master Mix (Life Technology, 4309155), GAPDH was used as the endogenous control during the real-time PCR process. The SYBR Green real-time PCR Master Mix, 7.5 µL, 1 µL of cDNA, 0.5 mL of Forward Primer (10 pmol), 0.5 µL of Reverse Primer (10 pmol), and 5.5 µL of RNase-free H2O were combined to create the PCR mixes in 15 µL volumes. Real-time PCR's thermal cycling profile was as follows: 10 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 20 s, and 59°C for 30 s (Table 1).

**Wound healing assay**

A 12-well plate containing 0.5×106 PC3 and DU145 cells was seeded, and the cells were left to form a confluent monolayer overnight. After scraping the monolayer with a 10 mL pipette tip to eliminate any
floating cells, washed 3 times with PBS and the monolayer was then transfected with each precursor miR (miR-377 and miR-scrambled). At random locations inside each well, the cells were imaged, then incubated for 24 hours at 37°C. The cells were then captured on camera at the chosen locations for 24 hours. The initial and ultimate wound zones were taken into consideration while analyzing the actual cell migration.

**Apoptosis assay**

One day before transfection, PC3 and DU145 cells (3×104 per well) were seeded into a 12-well plate. Following this, oligonucleotides, including pre-377 and miR-scrambled, were transfected using Lipofectamine 2000 (Life Technology, 11668019), by the manufacturer’s instructions, at a final concentration of 100 nM. According to the supplier’s instructions (Solarbio, CA1020), cells were collected after 48 hours and resuspended in a binding buffer containing annexin V-FITC and propidium iodide for flow cytometry analysis. Each sample was run in triplicate.

**Proliferation assay**

1000 cells per well were planted in a 96-well plate for the cell growth curve detection. Cells were rinsed with PBS and then MTT (Sigma, M2128) (5 µg/ml) was added after 72 hours after transfection. The cells were maintained in the CO2 incubator for 3 hours at 37°C. 100 µL of DMSO was then added to the mixture to dissolve the crystals. The purple-blue formazan dye’s spectrophotometric absorbance was assessed at 540 nm in a microplate reader (reference wavelength: 630 nm). At least three times each experiment was repeated.

**Cell cycle assay**

PC3 and DU145 cells were plated in 24-well plates at a density of 1 ×106 cells/mL to examine the impact of the miR-377 on the PCa cell cycle. The cells were transfected with scramble oligonucleotide or pre-miR-377 using Lipofectamine 2000 by the manufacturer’s instructions (Life Technology, 11668019). The cells were exposed to cold 70% ethanol for 24 hours at -20°C. After being washed, the cells were stained with propidium iodide (PI) solution and allowed to air dry for 30 minutes. The cell cycle was investigated using flow cytometry (BD Biosciences, San Jose, CA, USA).

**Statistical analysis**

The cell proliferation, apoptosis, cell cycle, cell migration, and real-time PCR studies were all performed at least three times. The mean and standard error are used to express results (SE). Data from each test were entered into GraphPad Prism V.9 for one-way ANOVA statistical analysis. Using the REST 2009 software, all real-time PCR data were examined and standardized for mRNA against GAPDH. P value < 0.05 was considered significant.

**Results**
miRNA-377 can target the 3′-UTR of MYC in prostate cancer cell lines

Using miRDIP (http://www.ophid.utoronto.ca/), miRwalk (http://www.mirwalk.umm.uniheidelberg.de/), and TargetScan 4.0 (http://www.targetscan.org/), we looked for miR-377 binding sites in the 3′-UTR of MYC. MiR-377 was linked to the MYC gene's 3′-UTR (Fig. 1a). To confirm these miR-target contacts, we cloned the MYC complementary site into psiCHECK-2TM to demonstrate that miR-377 targets MYC. PC3 and DU145 cells were co-transfected using this plasmid. miR-377 reduces luciferase activity in comparison to scramble, as indicated in (Fig. 1b). Transfecting miR-377 into PC3 and DU145 cell lines decreased luciferase activity to 48.4%±1.555 and 33.85%±1.099 (P < 0.05) respectively.

MYC mRNA downregulated by transfecting miR-377 in prostate cancer cell lines

The results of the differential expression analysis of MYC showed that its expression of it in tumor tissues increased significantly in patients. Also, expression analysis of miR-377 in prostate cancer tissues compared to normal tissues has shown down expression (Fig. 2a, b). We transfected pre-miR-377 or a scrambled oligonucleotide into PC3 and DU145 to verify the hypothesis that overexpression of miR-377 downregulates MYC mRNA expression in PCa cell lines, and we then evaluated levels of MYC mRNA by quantitative real-time PCR. As shown in (Fig. 2c), the overexpression of miR-377 caused reductions in the level of MYC mRNA in PC3 to 0.4787 ± 0.0802, and in DU145 to 0.3827 ± 0.0720.

Transfection of miR-377 into PCa cell lines inhibited cell migration

The wound healing experiment is depicted in (Fig. 3a, b). 24 hours after the cell monolayers were injured, 32.5% of PC3 cells and 22.4% of DU145 cells without transfection had filled in the cleared regions. The adhesive cells were made to move into the wound region more hardly after miR-377 transfection. The optimum inhibitory effects of miR-377 on PC3 and DU145 reached 31.7% and 17.2%, respectively. Cell migration was significantly reduced in both prostate cell lines after transfection.

Overexpression of miR-377 induced apoptosis in prostate cancer cell lines

The impact of miR-377 on apoptosis in PC3 and DU145 cells was assessed using annexin V and PI in flow cytometry. When compared to controls, the findings showed a rise in the number of cells going through early apoptosis. As shown in (Fig. 3c,d), control cells for this experiment included un-transfected cells and cells transfected with scrambled oligonucleotides. In cells transfected with miR-377 as compared to controls, the apoptosis ratio increased considerably. The percentage of early apoptotic cells increased from 1.05–18.3% in PC3 and from 1.66–13.4% in DU145 transfected with miRNA-377.
Overexpression of miR-377 inhibited cell proliferation in prostate cancer cell lines

An MTT test was used to comprehend miR-377's function in the proliferation of prostate cancer cell lines. In 96-well plates of PC3 and DU145 cells, miR-377 or scrambled oligonucleotides were transfected. The proliferation of these cells was assessed 48 hours after transfection in contrast to non-transfected and scrambled cells. In PC3 and DU145 cell lines, we showed that the miR-377 may considerably (P 0.05) slow down cell proliferation when compared to the scrambled and control groups (Fig. 3e). Three different experiments were conducted.

Overexpression of miR-377 inhibited the cell cycle of PCa Cell lines

To examine the miR-377 function on the cell cycle, we transfected miR-377 in PCa cell lines. According to the FCM analysis of the cell cycle, overexpression of miR-377 prevented cells from progressing through the G0/G1 phase, halting the proliferation of cancer cells (Fig. 4a,b).

Discussion

With more than 160,000 new cases each year, prostate cancer is the most frequently diagnosed malignancy in males. Prostate cancer is still the third-leading cause of cancer-related mortality in males, despite often taking an indolent course[18]. One of the most frequent chromosomal abnormalities in prostate cancer development that overexpressed in PCa has been identified to be alterations of chromosome 8, including amplification at 8q24 containing the MYC oncogene[19]. The proto-oncogene MYC's transcript is often overexpressed in prostate cancer (PCa). Most instances of advanced and metastatic castrate-resistant PCa also have elevated MYC protein abundance (mCRPC) [20].

The dysregulation of miRNA, a class of small endogenous regulatory RNAs with a size range of 17 to 27 nucleotides, is a key factor in the development of tumors[21]. Previous studies in pancreatic and lung cancer have shown that miR-377 has decreased expression in tumor samples compared to controls [11, 12]. Additionally, it has been shown that miR-377 expression is markedly downregulated in prostate cancer tissue and that this downregulation is linked to the proliferation, apoptosis, migration, and invasion of metastatic prostate cancer cells [13].

As has shown the downregulation of miR-377 in prostate cancer cells, in the current investigation, we concentrated on its impact on prostate cancer cell lines. In this study, we demonstrated that the 3′-UTR of MYC is a functional target region for miR-377 in PC3 and DU145 cells. The 3′-UTR of MYC is a functional target region for miR-377 in PC3 and DU145 cells, according to data from luciferase reporters. There is much research showing that many human cancers, including lung tumors, hepatocellular carcinoma[22], osteosarcoma MG-63[23], clear cell renal cell carcinoma[24], glioblastoma[9], malignant melanoma[25], pancreatic cancer[12], and ovarian cancer[26], have been associated to miRNA-377 downregulation.
We also examined the levels of MYC mRNA expression in PC3 and DU145 cells after miR-377 transfection, to confirm it could target this mRNA. Results indicated that miR-377 can decrease the MYC gene's mRNA expression level.

The effects of miR-377 on cell growth, apoptosis, and cell cycle were examined using an MTT test and flow cytometry, respectively. The findings showed that miR-377 significantly suppressed cell proliferation, induced apoptosis, stop the cell cycle at G0/G1 phase, and inhibit cell migration in PC3 and DU145 cells when compared to the control and scrambled miRNA, suggesting that miR-377 may have a function in preventing prostate cancer by targeting MYC 3'UTR. These findings were consistent with earlier research that showed miR-377 inhibits the proliferation of lung cancer, hepatocellular carcinoma, human osteosarcoma, clear cell renal cell carcinoma, glioblastoma, pancreatic cancer, and ovarian cancer cells while promoting apoptosis in pancreatic cancer and lung cancer cells.

According to all available information, miR-377 is essential for prostate and other cancer cells to proliferate and undergo apoptosis. There are still questions about the matching molecules engaged in the aforementioned processes and other miR-377 targets.

**Conclusion**

In conclusion, we discovered that miR-377 regulates MYC mRNA in prostate cancer cell lines by targeting its 3'-UTR. miR-377 transfection inhibits cellular proliferation, migration, and cell cycle and promotes apoptosis in PC3 and DU145 cells. Future research may discover more miR-377 targets to enhance the understanding of the miR-377 regulatory network that drives PCa development. Additionally, our findings suggest that the therapeutic target miR-377 for the treatment of prostate cancer may be promising.

**Declarations**

**Funding**

The Pasteur Institute of Iran provided funding for this research; however, the funders had no input on the study's planning, data collecting, analysis, the decision to publish, or manuscript preparation.

**Competing Interests**

All authors have no relevant financial or non-financial interests to disclose.

**Authors' contributions**

YA, FRJ and MA designed the study. MA, YA, and SH collected and analyzed the data. YA and MA wrote the manuscript. GA and SH discussed the review content and critically reviewed the manuscript draft. All authors read and approved the final manuscript.

**Data availability statement**
The authors confirm that the data supporting the findings of this study are available.

**Ethics approval**

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Pasteur Institute of Iran.

**Consent to participate**

Not applicable.

**Consent to publish**

Not applicable.

**References**


Tables

Table 1 Relative primers used for real-time PCR
<table>
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<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td>MYC</td>
<td>Forward: GTAGTCGAAAACCAGCCTCCC</td>
<td>116bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTCTCCTCCTCGTCGAGTA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: GAAAGCCTGCGGCTGACTAA</td>
<td>152bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCGGCAATACGACCAAATC</td>
<td></td>
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Figure 1

MYC is a direct target of has-miR-77. a) miR-377 has binding sites within the 3′-UTR of the human MYC gene. b) PC3 and DU145 cells were transfected with Renilla and firefly luciferase expression constructs psiCHECK KTM-2 harboring MYC 3′-UTR and miR-377 or scramble miR. Dual-Luciferase assays were done after 24 hours. The relative Renilla luciferase activity went down, but the scramble miR had no effect.
Figure 2

The level of MYC mRNAs following miRNA-377 or scrambled transfection. a) Analysis of MYC expression in PCa via TGCA samples. b) Analysis of miR-377 expression in PCa via TGCA samples. c) PC-3 and DU145 were transfected with miR-377 or scrambled. After 48 h, MYC expression was evaluated by real-time quantitative PCR. The decrease in relative mRNA expression was evident with miR-377, while no
effect was detected with the scrambled miR. Data represent mean ± SE from 3 independent experiments performed in triplicates.

Figure 3

The effects of the miR-377 on cell migration, apoptosis, and MTT of prostate cancer cell lines. a) Representative images from wound healing assay of PC-3 and DU145 cell cultures 24 h after miR-377
transfection compared to control cells. b) Wound healing assay graph of PC-3 and DU145 cell cultures 24 h after miR-377 transfection demonstrated that cell invasion into the cell-free region (outlined) is abatement compared to control cells. c) Apoptosis induction was investigated using flow cytometry. d) Apoptosis rates are shown in the graphs; Overexpression of miR-377 can induce apoptosis in PC3 and DU145 cell lines. e) The proliferation of PC3 and DU145 cells transfected with miRNA-377 scrambled oligonucleotide and control cells were determined using the MTT assay. Experiments were performed three times and data are shown as mean ± SD. The results showed that miRNA-377 could markedly inhibit cancer cell proliferation of PCa cell lines.
The effects of the miR-377 on the cell cycle of prostate cancer cell lines at the G0/G1 phase. a) PC-3 and DU145 cells were transfected with miR-377 and subjected to PI staining before flow cytometry. Cell cycle stop was investigated using flow cytometry. b) The graphs show that miRNA-377 could markedly stop the cell cycle at the G0/G1 phase of PCa cell lines compare to miR-scrambled and control.
Supplementary Files

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- GraphicalAbstract.jpeg