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microRNA-205 and microRNA-338-3p Reduces Cell Apoptosis in Prostate Carcinoma Tissue and LNCaP Prostate Carcinoma Cells by Directly Targeting the B-Cell Lymphoma 2 (Bcl-2) Gene

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Data Collection B
Statistical Analysis C
Data Interpretation D
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Background: The inhibitor of apoptosis, B-cell lymphoma 2 (Bcl-2), is encoded by the BCL2 gene. Previous studies have shown that microRNAs are downregulated in prostate cancer. This study aimed to investigate the role of microRNA-205 and microRNA-338-3p and cell apoptosis in prostate carcinoma tissue and the LNCaP human prostate adenocarcinoma cell line by directly targeting the BCL2 gene and Bcl-2 protein expression.

Material/Methods: Bioinformatics methods predicted the target genes of miR-205 and miR-338-3p, which were validated by a luciferase assay. Immunohistochemistry was used to detect Bcl-2 protein expression in 30 samples of prostate carcinoma tissue and 30 matched samples of normal prostate. The normal prostate epithelial cell line, RWPE-1, and LNCaP human prostate adenocarcinoma cells studied *in vitro*. BCL2 mRNA expression and Bcl-2 protein expression were determined by quantitative polymerase chain reaction (q-PCR) and Western blot, respectively. Cell apoptosis was measured by flow cytometry using annexin V, fluorescein isothiocyanate, and phycoerythrin (annexin V-FITC/PE).

Results: TargetScan Human 7.2 predicted that the structures of miR-205 and miR-338-3p had a binding site on the proto-oncogene, BCL2, which was verified by a luciferase assay. The expression of miR-205 and miR-338-3p were significantly downregulated in prostate carcinoma tissues and LNCaP cells when compared with normal controls. BCL2 expression was significantly inhibited by overexpression of miR-205 and miR-338-3p in LNCaP cells.

Conclusions: The results of this study showed that miR-205 and miR-338-3p downregulated the expression of the BCL2 gene and decreased apoptosis in prostate carcinoma.

MeSH Keywords: Apoptosis • Genes, bcl-2 • MicroRNAs • Prostatic Neoplasms

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Background

Epidemiological data of global cancer mortality showed that in 2013 prostate carcinoma was the second most common cancer in men [1]. There is still a need to identify potential diagnostic, therapeutic, and prognostic biomarkers to improve the prognosis for patients with prostate carcinoma [2,3]. Currently, the leading known risk factors for prostate carcinoma include age, race, and a family or genetic history of this malignancy [4]. It has been reported that the chromosome region 19q12-13 may be closely associated with the pathogenesis of prostate carcinoma [5–7]. The kallikrein-related peptidase 15 (KLK15) gene, which is adjacent to the KLK3 gene that encodes prostate-specific antigen (PSA) in the genome, is a recently cloned gene that has been shown to be associated with prostate carcinoma [8]. Studies have shown that KLK15 is significantly upregulated in prostate carcinoma [9,10].

It is now known that microRNAs (miRNAs) play an important role in the regulation of target genes. However, there have been few studies on the role of miRNAs in prostate carcinoma [11,12]. As a class of non-coding RNAs with a length of 22bp [13], miRNAs are of vital importance in biological processes, including cell differentiation and apoptosis, and there is increasing evidence to support that miRNAs are associated with human tumors [14–16]. Lorio et al. showed that miR-125b and miR-145 were significantly downregulated in breast cancer [17]. In 2015, Lan et al. proposed that miRNAs could be used as potential biological targets the treatment of human malignancy [18]. Studies on the role of miRNAs that are differentially expressed in cancer tissues are important because of their potential role in the development, metastasis, and chemotherapy resistance of malignant tumors.

Previously published studies have shown that the expressions of miRNAs are associated with the development of prostate carcinoma [19,20]. The expression of miR-1266-5p was shown to be significantly reduced in prostate carcinoma, which was associated with increased apoptosis in prostate carcinoma cells through activation of the BCL2 gene [21]. Studies have also shown that reduced expression of miR-4735-3p can promote apoptosis in prostate carcinoma during chemotherapy [22], and that both miR-711 and miR-221 have a role in the development of prostate carcinoma and can act as diagnostic markers [23,24]. The results of these previous studies indicate that miRNAs are not only biomarkers for the diagnosis and prognosis of prostate carcinoma, but are also associated with response to treatment.

Also, studies have shown that miR-205 inhibited cell growth and invasion in thyroid cancer by targeting the YAP1 tumor suppressor gene [25,26]. Also, miR-205 has been shown to affect cell migration, invasion, and epithelial-mesenchymal transition (EMT) by targeting the ZEB1 gene via the Akt/mTOR signaling pathway [27]. It has also been reported that miR-338-3p, which functions as a tumor suppressor, plays an important role in human malignancy, including gastric cancer, and osteosarcoma [28–31]. In liver cancer cells, reduced expression of miR-338-3p, which targets the sphingosine kinase 2 gene (SPHK2), has been shown to be associated with inhibition of tumor cell proliferation [32]. Also, downregulation of tumor tissue expression of miR-338-3p has been shown to be predictive of reduced prognosis in patients with gastric cancer [33].

The micro-RNA profiling data for GSE14857 and GSE60117 from the Gene Expression Omnibus (GEO) database has shown that the expression of miR-205 and miR-338-3p are decreased in prostate carcinoma tissue when compared with normal adjacent prostate tissue. However, the functional roles of miR-205 and miR-338-3p in prostate carcinoma remain unknown. Therefore, this study aimed to investigate the role of microRNA-205 and microRNA-338-3p and cell apoptosis in prostate carcinoma tissue and the LNCaP human prostate adenocarcinoma cell line by directly targeting the BCL2 gene and Bcl-2 protein expression.

Material and Methods

Ethical approval

All procedures involving human participants in this study were in accordance with local ethical standards and approved by the Institution Review Board of the Third Xiangya Hospital of Central South University, China. Patients who participated in the study provided informed consent.

Bioinformatics analysis

The micro-RNA profiling data for GSE14857 and GSE60117 from the Gene Expression Omnibus (GEO) bioinformatics database (*https://www.ncbi.nlm.nih.gov/geo/*) was used to obtain miRNA expression data for prostate carcinoma and normal tissues, with a false discovery rate (FDR) <0.05 and $\log_2$ fold change (FC) >1. The targets of miRNA were predicted by TargetScan Human 7.2 (*http://www.targetscan.org/vert_72/*) [34]. The network of target and miRNAs was analyzed by Cytoscape version 3.6.1 (*http://www.cytoscape.org/*) [35].

Cell culture and miRNA extraction

Cells from a normal prostate epithelial cell line, RWPE-1, and LNCaP human prostate adenocarcinoma cells were cultured with medium containing 10% fetal bovine serum (FBS) in an incubator containing 5% CO_2 at 37°C. Cultured cells were collected when they reached 90% confluence, and miRNA extraction was performed using the PureLink™ RNA Mini Kit (Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's instructions.

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Prostate tissue sample collection

Thirty clinical samples of prostate carcinoma and adjacent normal prostate tissues were collected from patients with prostate carcinoma undergoing prostate resection surgery at the Third Xiangya Hospital of Central South University, China. Participants were included in the study if, before tissue collection, the patients were not treated with chemotherapy, preoperative radiotherapy, or radiofrequency ablation. The tissue samples were immediately frozen in liquid nitrogen for further analysis. Demographic and clinicopathological data were obtained from medical records.

Quantitative polymerase chain reaction (q-PCR)

Total RNA of tissue samples was extracted with TRIzol reagent. After electrophoresis in a 1% agarose gel, the integrity of RNA and the RNA content were determined by ultraviolet (UV) spectrophotometry. Reverse transcription polymerase chain reaction (RT-PCR) was used to synthesize cDNA (Promega Corp., Madison, WI, USA). Using cDNA as a template, the mRNA expression levels of target genes were detected by qPCR using the specific primers for BCL2 and β-actin shown in Table 1. The reaction conditions were as follows: 5 min of predenaturation at 94°C, 30 s of denaturation at 94°C, 30 s of annealing at 58°C, 45 s of extension at 72°C, repeated for 28 cycles, followed by an 8 min extension at 72°C.

Cell transfection

The miR-205 mimics, miR-205 inhibitor, miR-338-3p mimics, and miR-338-3p inhibitor and the negative control were all purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The cells were transfected using 100 nm Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturer's instructions.

MTT assay

Cultured RWPE-1 and LNCaP cells in the logarithmic growth phase were seeded into a 96-well plate at a density of 5×10^3 cells per well. The MTT assay was used to detect cell viability after 0, 6, 12, 18, and 24 hours. The cells were incubated with 5 mg/ml MTT reagent (10 µl) for 4h, and the culture medium was discarded. Then, 100 µl of dimethyl sulfoxide (DMSO) was added to the cells and mixed until the crystals were dissolved. The optical density (OD) measurements for each well were determined at 490 nm using a microplate reader. Cell viability (percentage) was defined as: (the OD value in the treatment group – the OD value in the blank group)/(the OD value in the control group – the OD value in the blank group).

Table 1. List of primers used for BCL2 quantitative polymerase chain reaction (qPCR).

Primer name	Primer sequence (5'-3')
β-actin (forward)	5'ATGGGTCAGAA GGATTCCTATGTG3'
β-actin (reverse)	5'CTTCATGAGGTAGTCAGTCAGGTC3'
BCL2 (forward)	5'ATTGTGGCCTTCTTTGAGTTCG 3'
BCL2 (reverse)	5'CATCCCAGCCTCCGTTATCC 3'

Flow cytometry for apoptosis using the annexin V, fluorescein isothiocyanate, and phycoerythrin (annexin V-FITC/PE) detection method

Annexin V, fluorescein isothiocyanate, and phycoerythrin (annexin V-FITC/PE) staining was used to detect cell apoptosis. Briefly, cells were digested with 0.25% trypsin to obtain single cell suspensions, and the cell density was adjusted to between 5×10^6/ml and 1.0×10^6/ml. A volume of 1 ml cells was washed with phosphate buffered saline (PBS), and centrifuged three times at 1000 rpm at 4°C for 10 min. The supernatant was discarded, and the cell suspension and 200 µl binding buffer were added to 10 µl Annexin V-FITC and 5 µl of PE, and protected from light for 15 min after being mixed, followed by the addition of 300 µl of binding buffer. Flow cytometry was performed using an argon ion laser beam of 488 nm.

Immunohistochemistry for detection of Bcl-2 protein expression in prostate carcinoma tissue and normal prostate tissue

Immunohistochemistry was used to detect the expression of Bcl-2 protein in normal prostate tissue and tissue containing prostate carcinoma. Formalin-fixed, paraffin-embedded tissue sections were placed on a hotplate at 60°C for 30 min, dewaxed, and incubated for 20 min with H_2O_2 at room temperature, washed with distilled water, then rinsed three times with PBS (for 5 min each time). The sections were heated for 20 min in a water bath with antigen retrieval solution. After cooling to room temperature, the tissue sections were washed three times with PBS (for 5 min each time). The tissue sections were incubated with the primary antibody to Bcl-2 (1: 100 (clone no: ab117115) (Abcam, Cambridge, MA, USA) and incubated overnight in a humid chamber at 4°C. The sections were washed with three times in PBS (for 5 min each time), a secondary biotin-labeled antibody was added and incubated on the tissue sections for 20 min in a humid chamber at 37°C, and washed with three times in PBS (for 5 min each time). Horseradish peroxide (HRP)-labeled streptavidin was added and incubated on the tissue sections for 20 min in a wet box at 37°C. The tissue sections were washed and developed using

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the brown chromogen, 3,3'-diaminobenzidine (DAB), followed by washing in distilled water, counterstaining with hematoxylin, dehydration, mounting and the addition of a glass coverslip before evaluation by light microscopy.

Western blot for Bcl-2 protein

Cells in the logarithmic growth phase were selected, washed once with PBS, lysed with 200 µl 1% sodium dodecyl sulfate (SDS) to extract fresh protein. The protein was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, 50–100 µg of protein was transferred to polyvinylidene difluoride (PVDF) membranes after separation using 12% sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE) gels, and sealed for 1 h with Tris-buffered saline and Tween 20 (TBST) containing with 5% dried skimmed milk powder. Then, the protein was incubated with a primary antibody to Bcl-2 (Sigma-Aldrich Chemical Company, St Louis MO, USA) and incubated overnight at 4°C or for 2 h at room temperature, washed three times with TBST (for 5 min each time), incubated for 1.5 h with secondary antibodies at room temperature, and washed three times with TBST (for 10 min each time). Enhanced chemiluminescence (ECL) was used to develop the gels. ImageJ software was used for the analysis of the optical density (OD) values. β-actin was used as a control. The experiment was performed five times.

Plasmid construction and the luciferase assay

The 3'UTR sequence was downloaded from the prediction website (*http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi*). BCL2 (hs_nm_000633) 3'UTR was amplificated by PCR and then cloned into XbaI restriction site of the pGL3-control vector (Promega Corp., Madison, WI, USA).

Statistical analysis

All data quantification and statistical analysis were performed using SPSS version 17.0 software (SPSS, Chicago, IL, USA). Data were expressed as the mean ±SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) or two-tailed unpaired Student's t-test. A P-value <0.05 was considered to represent a statistically significant difference.

Results

miR-205 and miR-338-3p were significantly downregulated in prostate carcinoma tissues and cells

The micro-RNA profiling data for GSE14857 and GSE60117 from the Gene Expression Omnibus (GEO) database showed that they were 11 miRNAs that showed different expression between prostate carcinoma tissues and adjacent normal prostate tissues, and miR-338-3p and miR-205 were decreased in prostate carcinoma tissues (Table 2). Also, the expression of miR-338-3p and miR-205 found in clinical tissues and prostate carcinoma cells supported the results of the bioinformatics analysis. When compared with normal prostate tissues, the expression of miR-205 and miR-338-3p were significantly lower in prostate carcinoma tissues compared with normal prostate tissue. Compared with the normal prostate epithelial cell line RWPE-1, miR-205 and miR-338-3p expression were also significantly decreased in the LNCaP human prostate adenocarcinoma cells (Figure 1).

Table 2. Expression of different microRNAs in prostate carcinoma tissues compared with adjacent normal prostate tissues.

Acc ID	Prostate carcinoma *vs.* adjacent normal prostate
Has-miR-205	Down
Has-miR-149	Down
Has-miR-1274b	Up
Has-miR-1260	Up
Has-miR-1207-5b	Down
Has-miR-1274a	Up
Has-miR-142-3p	Up
Has-miR-1268	Down
Has-miR-338-3p	Down
Has-miR-14b-50	Up
Has-miR-21-5p	Up

Downregulation of miR-205 and miR-338-3p promoted prostate carcinoma cell growth

Compared with the normal control (NC) group, the growth of prostate carcinoma cells *in vitro* was significantly enhanced in the cells transfected with the miR-205 inhibitor and inhibited in the cells transfected with the miR-205 mimics. Also, cell growth of the prostate carcinoma cells was significantly promoted in the cells transfected with miR-338-3p inhibitor, and significantly inhibited in the cells transfected with miR-338-3p mimics. These results indicated that miR-205 and miR-338-3p had similar functions, and both could reduce the growth of prostate carcinoma cells (Figure 2).

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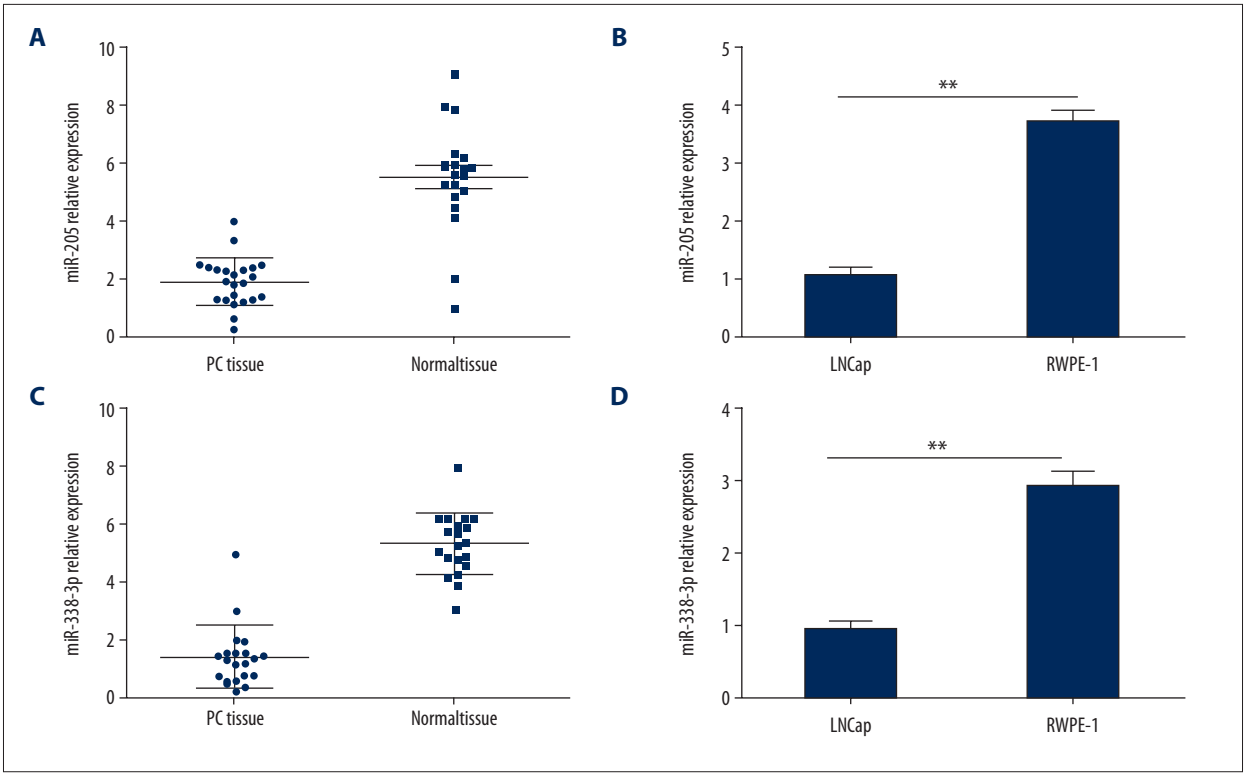

Figure 1. Expression of micro-RNAs, miR-205, and miR-338-3p in the prostate tissues and LNCaP human prostate adenocarcinoma cells. **A** and **C** show that miR-205 and miR-338-3p expression in cancer tissues was significantly lower when compared with normal tissues. **B** and **D** show that miR-205 and miR-338-3p expression in LNCaP human prostate adenocarcinoma cells was significantly lower when compared with normal cells. PC – prostate carcinoma. ** p<0.01 when compared with normal.

miR-205 and miR-338-3p promoted prostate carcinoma cell apoptosis

The miR-205 mimics, miR-205 inhibitor, miR-338-3p mimics, miR-338-3p inhibitor, and corresponding controls were transfected into prostate carcinoma cells, and cell apoptosis was measured by flow cytometry using annexin V, fluorescein isothiocyanate, and phycoerythrin (annexin V-FITC/PE). Compared with the control group, prostate carcinoma cell apoptosis was inhibited in the cells transfected with miR-205 inhibitor or miR-338-3p inhibitor and was promoted in the cells transfected with miR-205 mimics or miR-338-3p mimics. These results indicated that miR-338-3p and miR-205 also inhibited prostate carcinoma cell apoptosis (Figure 3).

Increased expression of the BCL2 gene and Bcl-2 protein in prostate carcinoma

Targetscan predicted that the structures of miR-205 and miR-338-3p had a binding site on the proto-oncogene, BCL2 (Figure 4A). To test whether BCL2 was a direct target gene of miR-205 and miR-338-3p, wild-type or mutated plasmid or a negative control were co-transfected with miR-338-3p mimics into prostate carcinoma cells. The luciferase assay showed that, compared with the control group, the plasmid activity was significantly decreased after co-transfection with miR-338-3p mimics and wild-type (WT) plasmid. Compared with the negative control, there was no significant difference between the WT plasmid or mutated vector ($P < 0.05$), and miR-205 showed similar results (Figure 4B, 4C). These results indicated that miR-205 and miR-338-3p could regulate the expression of BCL2 by direct targeting of BCL2 mRNA. The expression of the Bcl-2 protein was mainly expressed in the cytoplasm of prostate carcinoma cells and minimally expressed in normal prostate epithelial cells detected by immunohistochemistry (Figure 4D, 4E).

miR-205 and miR-338-3p significantly affected the expression of BCL2

To further investigate the effect of miR-205 and miR-338-3p on the BCL2 gene, the expression of BCL2 was detected in tumor cells transfected with miR-338-3p mimics and inhibitor. The results showed that the expression of BCL2 was downregulated after transfection with miR-338-3p mimics and increased after transfection with miR-338-3p inhibitors (Figure 5). Similar results were also shown in cells transfected with miR-205. These results indicated that miR-205 and miR-338-3p negatively regulated the expression of BCL2.

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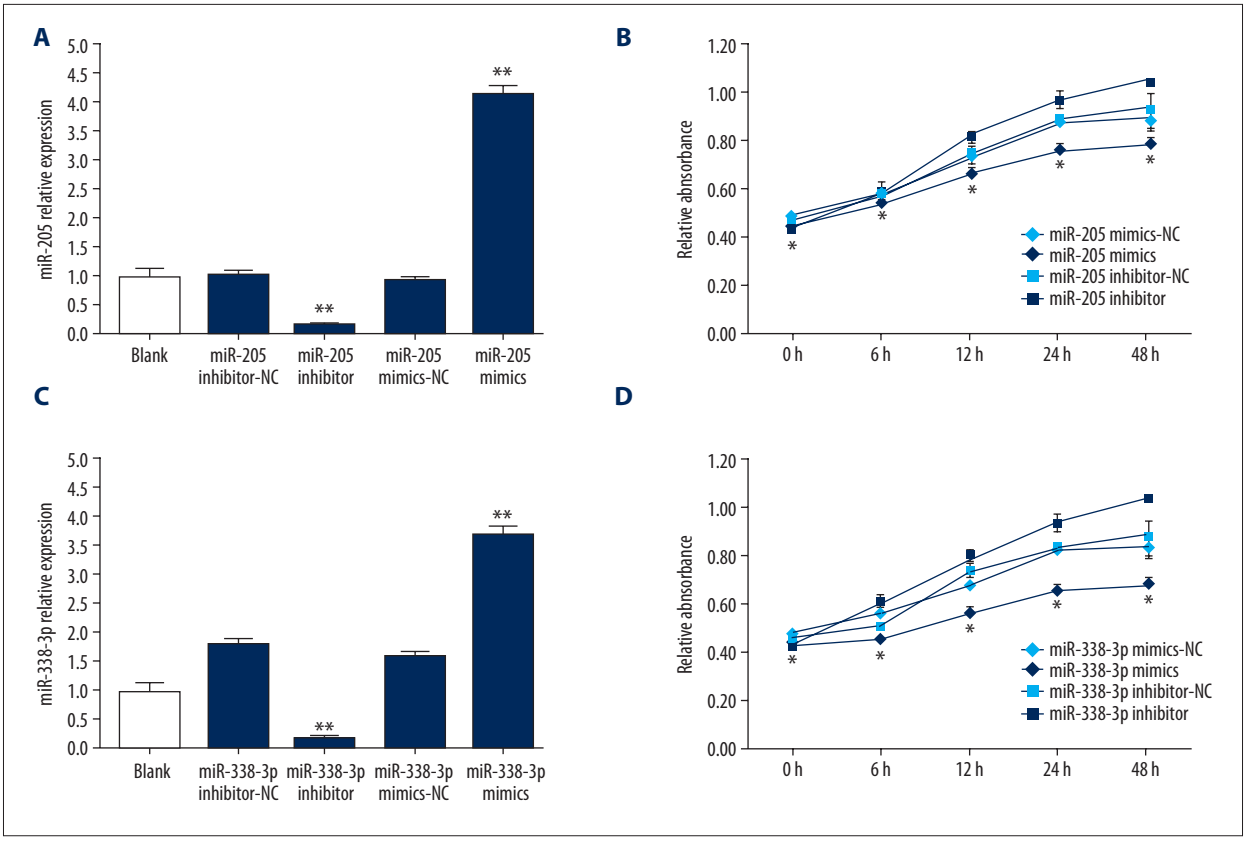

Figure 2. Growth of LNCaP human prostate adenocarcinoma cells after transfection. **A** and **B**. Growth of LNCaP human prostate adenocarcinoma cells after transfection with miR-205. **C** and **D**. Growth of LNCaP human prostate adenocarcinoma cells after transfection with miR-338-3p. The results showed that the growth of the LNCaP cells was significantly inhibited by upregulation of miR-205 and miR-338-3p expression, and increased by inhibition of miR-205 and miR-338-3p expression. ** p<0.01 when compared with NC.

Discussion

As one of the key regulatory factors in tumors, microRNAs (miRNAs) have gained increasing attention. Recent studies have shown that miR-205 and miR-338-3p act as tumor suppressors and have a role in regulating the processes of tumor cell migration and apoptosis. For example, miR-38-3p has been shown to suppress cell proliferation and induce apoptosis of non-small cell lung cancer (NSCLC) by targeting sphingosine kinase 2 (SPHK2) and insulin receptor substrate 2 (IRS2) genes [36,37].

In hepatic stellate cells (HSCs), the expression level of miR-338-3p has previously been shown to be significantly decreased in HSCs, and overexpression of miR-338-3p has been shown to inhibit the expression of Col 1 and α-SAM, which are two major HSC activation markers, resulting in suppression of the proliferation of HSCs [38]. Recently, Hua et al. have shown that miR-338-3p was significantly decreased in cervical cancer tissues and overexpression of miR-338-3p could significantly inhibit cervical cancer cell proliferation and induce cell apoptosis through the MACC1/MAPK signaling pathway [39]. A further study has shown that miR-205 inhibited the expression of LRRK2 and induced cell apoptosis [40]. Nagai et al. found that miRNA-205 could suppress the invasive ability of oral squamous cell carcinoma by targeting TIMP-2 [41]. These results indicate that miR-338-3p and miR-205 have significant effects on proliferation, invasion, and apoptosis of tumor cells. In the present study, the expressions of miR-338-3p and miR-205 in prostate carcinoma cells were found to be significantly decreased in prostate carcinoma tissues and cells, which inhibited the proliferation and promoted the apoptosis of prostate carcinoma cells. However, the mechanisms of miR-205 and miR-338-3p in prostate carcinoma remained unclear, and the regulatory targets in prostate carcinoma still remained to be investigated.

Bioinformatics methods have provided new ways to predict the regulatory targets of miRNAs. In the present study, the TargetScan database was used to predict the potential targets of miRNA, and a large number of potential targets for miR-205 and miR-338-3p were obtained [34]. The results showed

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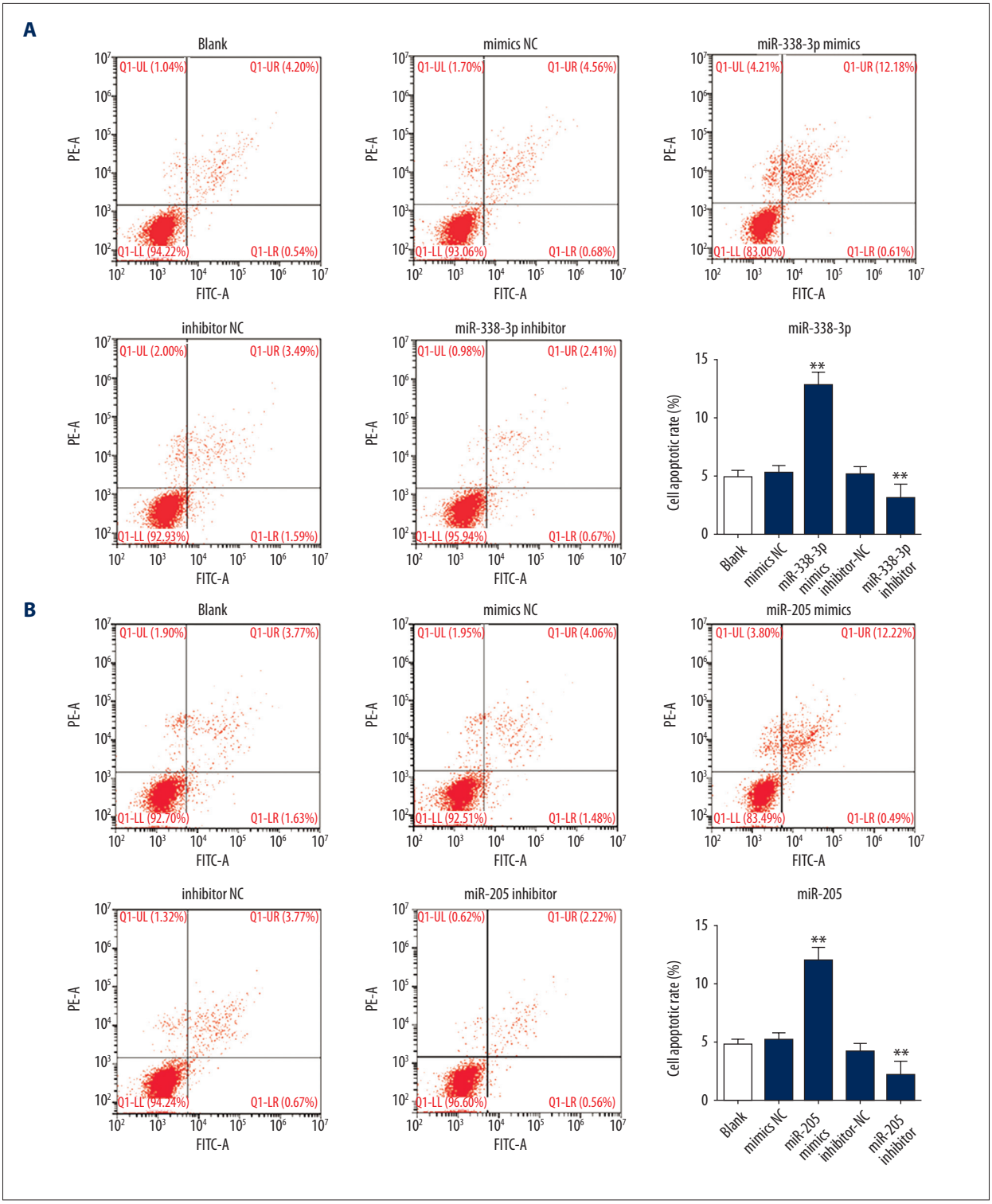

Figure 3. Apoptosis of LNCaP human prostate adenocarcinoma cells after transfection. (**A**) Apoptosis of LNCaP human prostate adenocarcinoma cells was promoted after transfected with miR-338-3p mimics and inhibited after transfected with miR-338-3p inhibitor. (**B**) Apoptosis of LNCaP human prostate adenocarcinoma cells was promoted after transfected with miR-342-5p mimics and inhibited after transfected with miR-342-5p inhibitor. ** p<0.01 when compared with NC.

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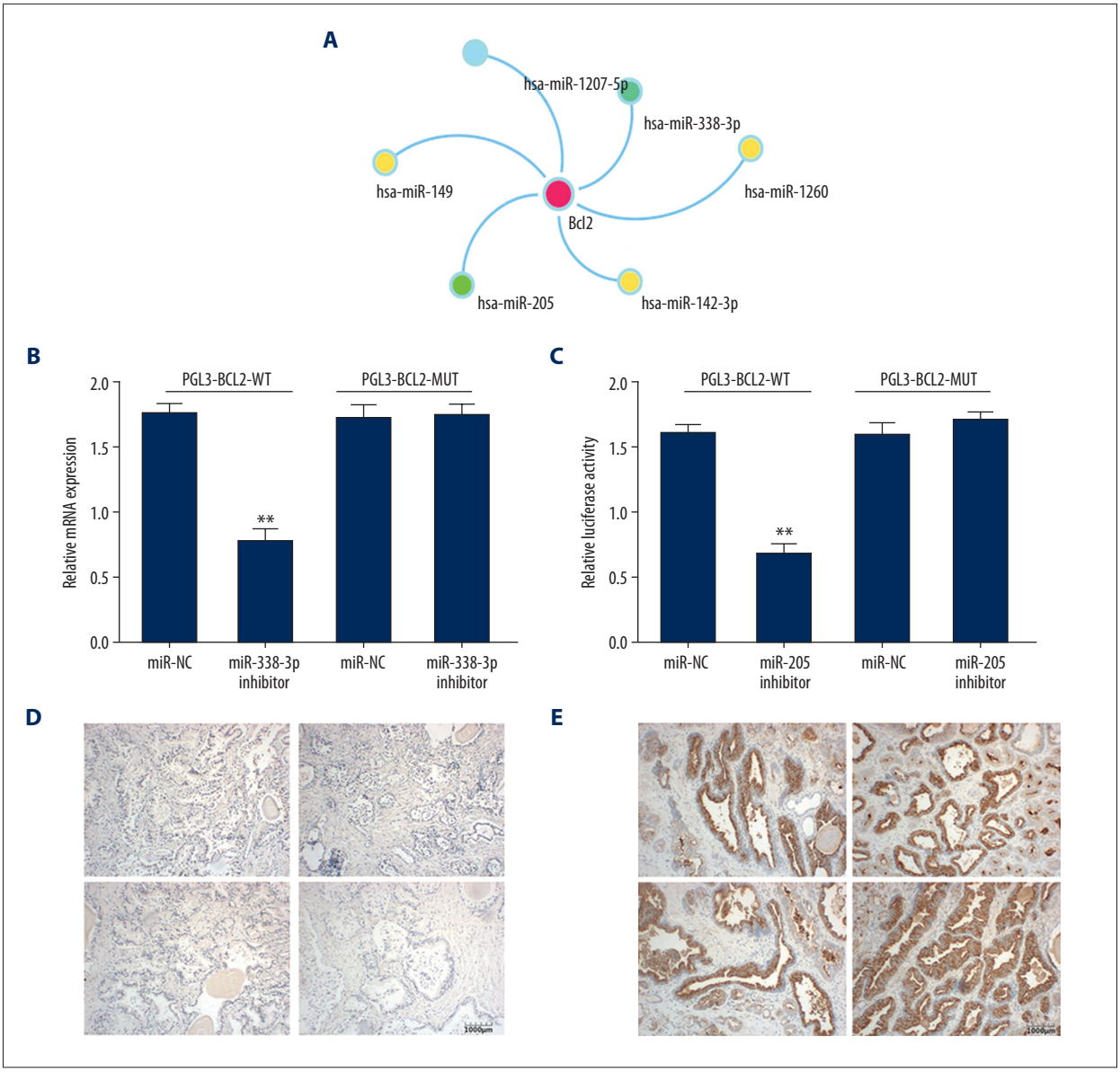

Figure 4. Expression of the BCL2 gene in prostate carcinoma tissues and normal prostate tissues. (**A**) MicroRNAs targeted by the BCL2 gene, from Targetscan bioinformatics. (**B**) The result of luciferase activity showed a direct interaction between miR-205 and miR-338-3p and the BCL2 gene. (**C**) Expression of BCL2 in normal prostate epithelial tissues. (**D**) Expression of BCL2 in prostate carcinoma tissues. PC – prostate carcinoma. ** p<0.01 when compared with NC.

that both miR-205 and miR-338-3p were targeted by the BCL2 gene. BCL2 plays a key role in modulating cell apoptosis and is also considered to be an oncogene [42]. The prognostic effect of BCL2 expression in breast cancer has been reported in several studies. For example, apoptosis of breast cancer cells has been shown to be significantly inhibited by BCL2 [43–45]. BCL2 is a major anti-apoptotic factor and is directly involved in the inhibition of cell apoptosis [46]. Khor et al. found that expression levels of BCL2 in prostate carcinoma cells was markedly increased [47]. The findings from these previous studies support those of the present study, which showed that BCL2 has an important role in tumor cell apoptosis, and support the use and accuracy of the use of bioinformatics methods for screening the targets of miRNAs.

The miRNAs reduce the expression of downstream genes by downregulating or degrading the target genes [48]. However, this study found that miR-205 and miR-338-3p were negatively correlated with BCL2 expression. Some previous studies have shown that miRNAs can interact with the gene promoters, thereby activating the transcription of genes, which further leads to the activation of downstream genes. There is

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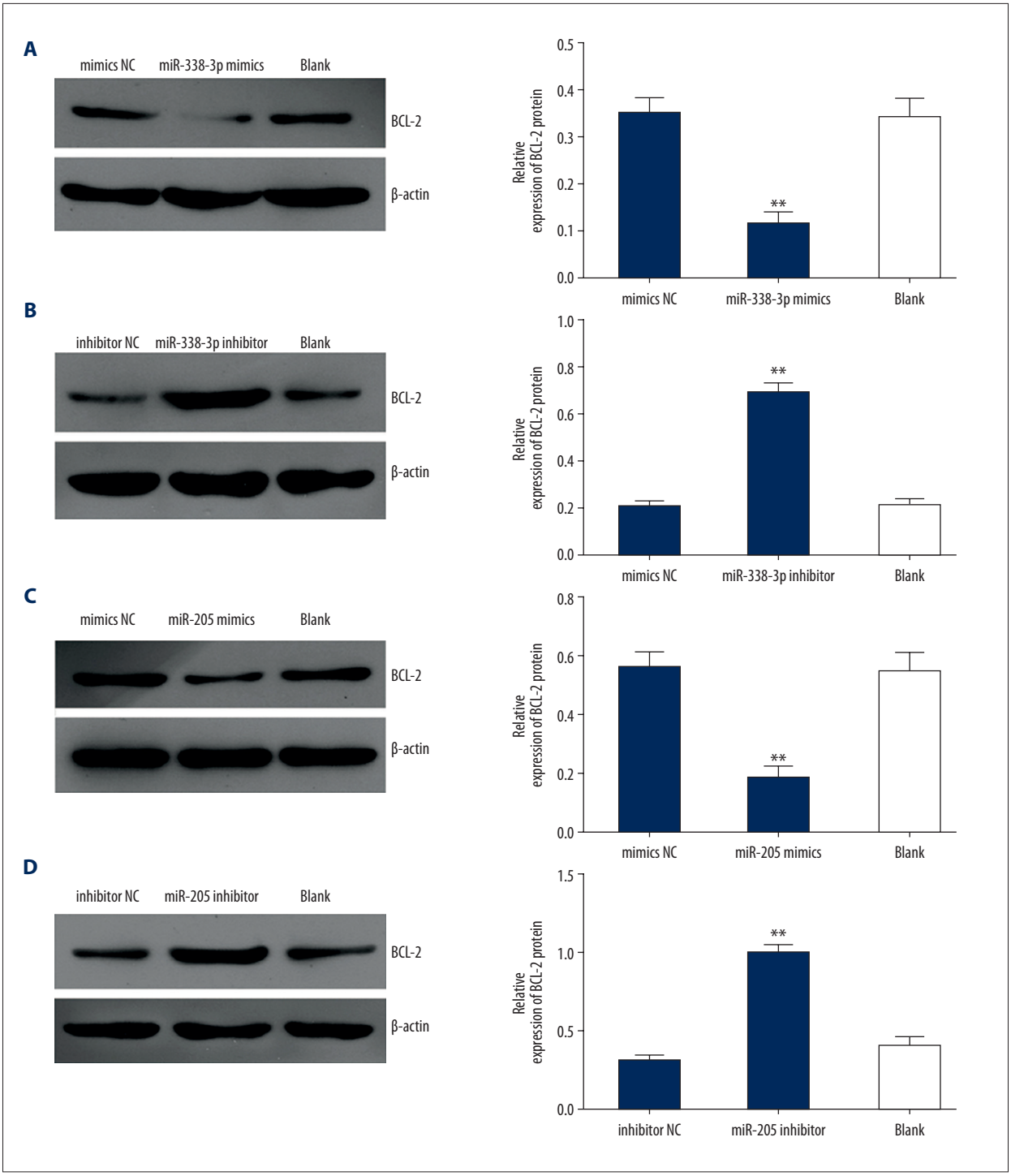

Figure 5. Micro-RNAs, miR-205, and miR-338-3p significantly increased the expression of the BCL2 gene. **A** and **B** show that inhibition of miR-338-3p significantly upregulated the expression of the BCL2 gene. **C** and **D** show that the inhibition of miR-205 significantly upregulated the expression of the BCL2 gene. ** p<0.01 when compared with the NC.

also a more generally accepted view that miRNAs have an effect on the 3-UTR gene, thereby inhibiting gene transcription or promoting the degradation of mRNA. The class of small RNA molecules known as small activating RNAs (saRNAs) and act by RNA activation (RNAa), as opposed to RNA interference (RNAi)) [49,50]. In 2008, Robert et al. showed that miR-373 could promote gene expression by combining with the promoters of the CSDC2 gene [51]. Although it has been reported that

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miRNAs can promote gene expression, because miRNAs may regulate several target genes, and the upstream suppressors of the target genes may also be regulated by miRNAs, this results in increased expression of target proteins. However, the findings of the present study showed the expression of the BCL2 gene and Bcl-2 protein were directly regulated by miR-205 and miR-338-3p.

Conclusions

The findings of this study showed that the expressions of miR-205 and miR-338-3p in prostate carcinoma cells were significantly lower compared with normal prostate cells. The BCL2 gene was identified as the direct target of miR-205 and miR-338-3p by bioinformatics methods, and verified through subsequent *in vitro* studies and validated by a luciferase assay. Also, miR-205 and miR-338-3p were shown to promote cell proliferation and inhibit the apoptosis of prostate carcinoma cells by negatively regulating BCL2. These findings indicate that miR-205 and miR-338-3p may provide a novel therapeutic strategy for the treatment of prostate carcinoma.

Conflict of interests

None.

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