

miR-1266-5p and miR-185-5p Promote Cell Apoptosis in Human Prostate Cancer Cell Lines

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Abstract

Objective: Small non-coding RNA molecules are dysregulated in prostate cancer (PCa). In our previous study, downregulation of miR-1266 and miR-185 was demonstrated in PCa tissues and cell lines. The aim of the present study was to investigate whether miR-1266 and miR-185 are involved in the regulation of B-cell lymphoma (BCL) 2 and BCL2L1, respectively, and whether transfection of PCa cell lines with miR-1266 and miR-185 mimics can alter tumorigenic phenotypes. **Methods:** In order to investigate the regulation of BCL2 and BCL2L1 mRNA levels by miR-1266 and miR-185, respectively, a luciferase reporter assay was used. Real-time PCR was also used to analyze changes in the levels of BCL2 and BCL2L1 mRNAs in PCa cell lines following transfection with synthetic miR-1266 and miR-185. Cell apoptosis was determined by Annexin V protein expression analysis via flow cytometry. In addition to the MTT assay, a cell proliferation assay was performed. **Result:** A luciferase assay confirmed that the BCL2 and BCL2L1 genes may be targeted by miR-1266 and miR-185, respectively, through binding to their 3'UTR regions. Transfection of PC3 and DU145 cells with miR-1266 and miR-185 induced apoptosis and reduced proliferation, which also revealed an inverse correlation with BCL2 and BCL2L1 gene expression in the treated cells. **Conclusion:** Our data suggests that miR-1266 and miR-185 may be novel candidates for further research in PCa treatment through the anti-apoptotic pathway.

Keywords: miR-1266-5p- miR-185-5p- prostate cancer- BCL2- BCL2L1

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Introduction

Prostate cancer (PCa) is the second leading cause of cancer-associated male mortality in the United States (Siegel et al., 2013), despite recent advances in the clinical management of patients. In 2015, the American Cancer Society announced that more than 220,000 new patients were diagnosed with PCa, among whom 25,000 would eventually succumb to the disease (Siegel et al., 2015). The rate of PCa in Iran has been reported to be 8.5% of all cancer types (Mohagheghi et al., 2009). However the trend of affection has been decreased over the course of past few decades (Kelly et al., 2017).

Apoptosis is an important component of cell growth control, and plays a key role in cell proliferation and death. Disturbances in the well-adjusted balance of apoptosis may contribute to cancer development (Sellers and Fisher, 1999; Wong, 2011; Hassan et al., 2014; Koff et al., 2015). Resistance to apoptosis was identified as one of the ten

hallmarks of cancer in 2011 (Hanahan and Weinberg, 2011; Hainaut and Plymoth, 2013), and apoptosis was found to be a natural pathway for protecting cells from malignant transformation (Adams and Cory, 2007). Apoptosis may occur via two pathways, namely 'extrinsic' and 'intrinsic' (mitochondrial) (Asadi et al., 2018a). The extrinsic pathway involves receptors on the cell surface, while the mitochondrial apoptosis pathway is stimulated by DNA damage or cytotoxic substances, and is regulated by B-cell lymphoma (BCL) 2 family proteins (Adams and Cory, 2007).

BCL2 family proteins are located on the outer membrane of the mitochondria, are known as gatekeepers in the apoptotic response, and play a key role in the control of permeabilization of the mitochondrial membrane. The BCL2 family members were identified as inhibitors of cell death and regulators of apoptosis. Drugs acting as BCL2 inhibitors appear to be promising approach in current clinical trials to control malignancies (Delbridge

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et al., 2016). BCL2L1 is a member of the BCL2 protein family that is transcribed into various isoforms. One of the longest isoforms, which is translated into a 233-amino acid protein with anti-apoptotic function is BCL2L1 (Revil et al., 2007).

The first microRNA was identified in 1993 through a genetic study on *C. elegans* (Lee et al., 1993; Hanahan and Weinberg, 2011). MicroRNAs are small non-coding RNAs (18-25 nucleotides in length), which typically bind to the 3'-untranslated region (3'-UTR) of mRNAs, leading to mRNA degradation (Doench and Sharp, 2004; Hanahan and Weinberg, 2011). Over the last decade, it has been found that non-coding RNAs, particularly microRNAs, are involved in cancer development (Rossi et al., 2012; Davudian et al., 2016; Mansoori et al., 2017; Asadi et al., 2018b). Their pivotal role as tumor suppressors or oncogenic factors has been previously reported (Hagman et al., 2010). miRNA profiling is a useful approach in distinguishing cancer types originated from various developmental lineages (Lu et al., 2005). In human PCa, miRNAs play an important role in cancer development by affecting cell apoptosis and proliferation (Casanova-Salas et al., 2012; Zhang et al., 2014; Wang et al., 2015).

Downregulation of miR-1266 and miR-185 was demonstrated in our previous study on PCa tissues and cell lines (Ostadrahimi et al., 2018). Selection of candidate microRNAs was first performed using bioinformatics prediction tools and a literature review. Subsequent expression analysis revealed a correlation between the downregulation of miR-1266 and miR-185, and the upregulation of BCL2 and BCL2L1, respectively.

The aim of the present study was to investigate the effects of the introduction of miR-1266 and miR-185 mimics in PCa cell lines on the levels of BCL2 and BCL2L1, in addition to cancer phenotypes, such as cell proliferation and apoptosis. The functional effects of miR-1266 and miR-185 on their targets was also investigated by the luciferase assay.

Materials and Methods

PCa cell lines, cell culture and reagents

The PC3 and DU-145 human PCa cell lines were purchased from the Leibniz-Institute DSMZ (Germany). DU-145 cells were cultured in 90% RPMI-1640 + 10% heat-inactivated (h.i.) FBS (Gibco, MA, USA). PC3 cells were cultured in 45% Ham's F12 + 45% RPMI-1640 + 10% h.i. FBS (Gibco, MA, USA). All cells were incubated in 5% CO₂ at 37°C. miR mimics (MIMAT0005920, MIMAT0000455 and MIMAT0000255), AllStars Negative Control siRNA, miScript II RT Kit, QuantiTect SYBR Green PCR Kit and miRNeasy Mini Kit were purchased from Qiagen GmbH (Hilden, Germany). Lipofectamine® 2000 was purchased from Invitrogen.

microRNA transfection

miR-1266-5p and miR-185-5p mimics were used for transfection of cell lines. A miR-34a mimic was used, as its effects on promoting apoptosis are well known. miR-Scrambled (Qiagen, Hilden, Germany) was used as

the negative control; however, preliminary data showed high toxicity of miR-Scrambled on cultured cells (even at minimal concentrations), thus, it was removed from the assay. Lipofectamine® 2000 was used for microRNA mimic transfection according to the manufacturer's protocol for 10,000 cells seeded in a 96-well plate. First, the culture medium was removed and replaced with fresh medium. Then, a mixture of 0.1 µl of each mimic in 0.3 µl Lipofectamine® 2000 diluted in 10 µl opti-MEM medium was added to each well. After 6 h, the medium was changed.

Cell viability

For the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was dissolved in phosphate-buffered saline (PBS) at a concentration of 3 mg/ml and the solution was filtered through a 0.45-µm pore membrane for sterilization. Cells (2x10³) were dispensed in each well of the 96-well plate and were transfected with miR-1266-5p (0.1 µl), miR-185-5p (0.1 µl), and miR-34a-5p (0.1 µl). At 24 h after transfection, MTT solution (11 µl) was added to each well containing 110 µl cultured medium for 4 h in 37 °C. Subsequently, to dissolve formazan crystals, solubilization solution (isopropanol 200 ml + HCL 1.66 ml; Sigma-Aldrich) was added. The absorbance was read with a spectrophotometer (A260/280 >2.0, A260/230 >1.8), using a NanoDrop ND-2000.

RNA extraction and cDNA synthesis

Cell lines: At 30 h after transfection with microRNA mimics, the total RNA of the cells was extracted using miRNeasy Mini kit. In summary, the cell lines were seeded to ~90% confluence (~100,000 cells/well into 24-well plates for 24 h), and were then disrupted by adding Qiazole (Qiagen, Hilden, Germany), after which the samples were separated into three phases by adding chloroform (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Ethanol 70% (Merck, Germany) was added to the aqueous phase to precipitate the RNA. Finally, after washing and centrifuging, total RNA was extracted, according to the manufacturer's instructions. The miScript II RT Kit (Qiagen, Hilden, Germany) was used for reverse transcription of RNA molecules to synthesize complementary DNA (cDNA), according to the manufacturer's instructions.

Quantitative PCR (qPCR)

First, cDNA was subjected to Real-time PCR using the QuantiTect SYBR Green PCR kit. A total of 20 µl of the reaction mixture were used, containing forward and reverse primers for BCL2, BCL2L1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, the housekeeping gene; Table 1). The cycling parameters were set as the following: 1 cycle at 95°C for 15 min, followed by 40 cycles at 94 °C for 15 sec, at 60 °C for 30 sec, and at 72 °C for 30 sec. All primers were synthesized by TAG Copenhagen A/S. The analysis of Real-time PCR data was performed using the 2^{-ΔΔCt} method.

Cell death rate (flow cytometry)

For flow cytometry analysis, 1×10^5 DU145 and PC3 cells were cultured in 12-well plates at a density of 5×10^5 cells per well. The culture medium was replaced after 24 h, and transfection with miR-185-5p, miR-1266-5p and miR-34 was performed. The cells transfected with blank Lipofectamine® 2000 were considered as negative controls.

To evaluate the extent of apoptosis and necrosis, the FITC Annexin V/Dead Cell Apoptosis Kit with FITC Annexin V and PI (Invitrogen) (for DU145) or the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen, Stockholm, Sweden) (for PC3) were used 24 h after cell transfection. A cell pellet was obtained from the centrifuged harvested cells of each well and washed with cold PBS, followed by addition of 5 μ l Annexin V and 1 μ l of the 100 μ g/ml PI (for DU145), or 5 μ l PE Annexin and 5 μ l 7-ADD (for PC3), to 100 μ l of cell suspension containing 1×10^5 cells. Following incubation for 15 min at 25 °C, 400 μ l binding buffer was added to each tube for analysis using a FACS Array flow cytometer (Becton Dickinson, San Jose, CA, USA) at the indicated wavelengths (FITC: excitation/emission wavelength, 550/573 nm; 7-ADD: excitation/emission wavelength, 488/674 nm).

Luciferase assay

For the luciferase assay, the 3'-UTR regional sequences of BCL2 (4507-4656, NM_000633.2) and BCL2L1 (region 2124-2274, NM_138578.2) were synthesized (Biomatiks) and sub-cloned into the XhoI/NotI poly-cloning site of psiCHECK™-2 Vector (Promega, C8021). The vectors were transformed and propagated in the DH5 α E. coli strain (Thermo Fisher Scientific, Inc.) and extracted using Plasmid Plus Maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

The amounts of luciferase produced by transformed plasmids were analyzed using Dual-Glo luciferase assay (Promega). Briefly, 1×10^5 cells were seeded in 12-well plates and incubated in 5% CO₂ at 37 °C for 24 h. After replacing the culture medium, 3 μ l miRNA and 100 ng vector were co-transfected into PC3 cells using 3 μ l Lipofectamine® 2000. After 8 h of incubation (obtained by optimizing the time), the luciferase assay was performed.

The tests were performed in triplicate.

Statistical analysis

Differences between groups were analyzed using a t-test with Welch's correction. Results are presented as means \pm standard deviation (SD). P-values of <0.05 were considered statistically significant. GraphPad Prism 5 was used for statistical analysis and drawing the graphs (GraphPad Software, La Jolla, CA, USA; <https://www.graphpad.com/>).

Results

Cell viability

The results obtained from the MTT assay are shown in Figure 1. The reduction in viability observed in the PC3 and DU145 cell lines 30 h after transfection with miR-1266-5p and miR-185-5p was considered to be significant. miR-34a was used, as previous studies had demonstrated its suppressive effect on cell viability. Cells receiving only Lipofectamine (blank) were used as the negative controls, and cells with no transfection were used as the external control group.

Expression analysis in the PC3 and DU145 cell lines

The BCL2 gene did not exhibit an expression reduction following miR-1266-5p transfection in the DU145 cell line. However, after miR-185-5p transfection in DU145 cells, the expression of BCL2 was reduced. miR-34a was transfected as a positive control, and resulted in reduction of BCL2 gene expression.

There was no significant reduction in the expression of the BCL2L1 gene following transfection with miR-185-5p or miR-1266-5p. Similarly, no significant reduction of BCL2L1 expression was observed following

Table 1. Forward and Reverse Primers for BCL2, BCL2L1, and GAPDH Genes Used for RT-PCR.

Gene	Sequence
BCL2 (F)	5'-GGATCCAGGATAACGGAGGC-3'
BCL2 (R)	5'-GGCAGGCATGTTGACTTCAC-3'
BCL2L1 (F)	5'-CCTTGGATCCAGGAGAACGGC-3'
BCL2L1 (R)	5'-GGGAGGGTAGAGTGGATGGTC-3'
GAPDH (F)	5'-AACGGGAAGCTGTGCATCAATGGAAA-3'
GAPDH (R)	5'-GCATCAGCAGAGGGGGCAGAG-3'

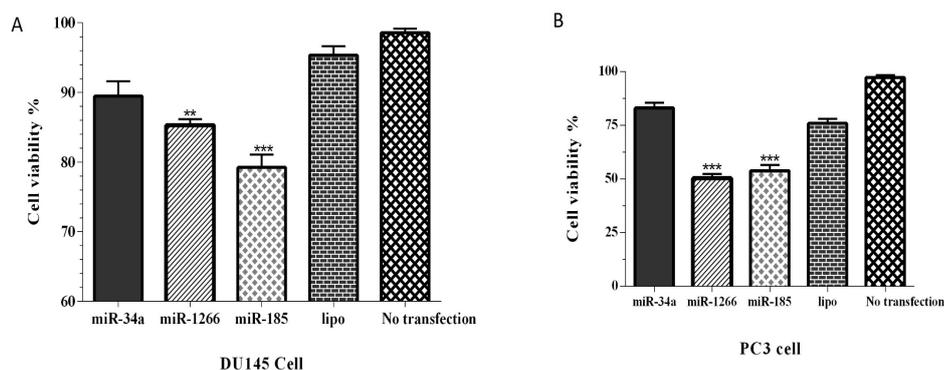


Figure 1. Cell Viability by MTT Assay after 30 hours. Transfection of DU145 (A) and PC3 (B) cell lines with microRNA-34a, microRNA-1266, microRNA-185, blank lipofectamin and no transfection.

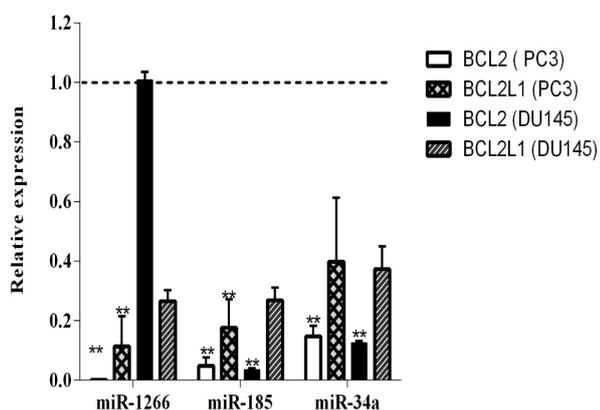


Figure 2. Relative Expression of BCL2 and BCL2L1 Genes 30 hours after Transfection of microRNA-1266, microRNA-185 and microRNA-34a in PC3 and DU145 Cell Lines. GAPDH gene was used as normalizer

miR-34a transfection in DU145 cells (Figure 2).

Although the bioinformatics results from www.microRNA.org revealed a complementary seed region between miR-34a and BCL2L1, to the best of our knowledge, no studies have demonstrated a correlation between miR34-a and BCL2L1 to date. In the present study, we did not observe a suppressor effect of miR-34a on BCL2L1 expression.

The BCL2 mRNA level was significantly decreased following transfection of PC3 cells with miR-1266-5p and miR-185-5p, as well as miR-34a (positive control). The BCL2L1 mRNA level was also significantly reduced following transfection with miR-1266-5p or miR-185-5p. However, miR-34a transfection in PC3 cells did not lead to any significant changes in BCL2L1 mRNA expression (Figure 2).

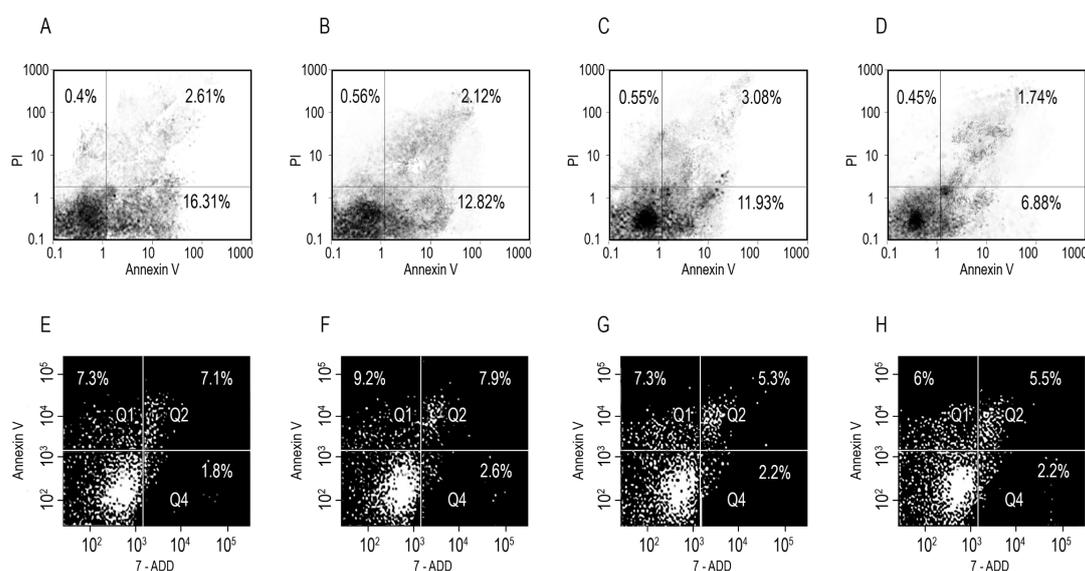


Figure 3. Annexin V Expression (Apoptosis Rate) Detection after Treatment by microRNA-1266-5p (A), microRNA-185-5p (B), microRNA-34a (C) and Lipofectamine (D) in DU145 Cell Line Using Flow Cytometry Method. Living cells are seen in left lower quadrant, Annexin V (-)/ PI (-), [Q1]. The early apoptotic cells are shown in right lower quadrant, Annexin V (+)/ PI (-), [Q2]. Advanced apoptotic or necrotic cells are seen in right upper quadrant, Annexin V (+)/ PI (+), [Q3]. The last quadrant Annexin V (-)/ PI (+), [Q4], may be bare nuclei, cells in late necrosis or cellular debris.

Annexin V expression (apoptosis rate) detection after treatment by microRNA-1266-5p (E), microRNA-185-5p (F), microRNA-34a (G) and Lipofectamine (H) in PC3 cell line using flow cytometry method. Living cells are seen in left lower quadrant, Annexin V (-)/7-ADD (-), [Q1]. The early apoptosis are shown in the left upper quadrant, Annexin V (+)/7-ADD (-), [Q2]. Advanced apoptotic or necrotic cells are seen in right upper quadrant, Annexin V (+)/7-ADD (+), [Q3]. The last quadrant Annexin V (-)/7-ADD (+), [Q4], may be bare nuclei, cells in late necrosis or cellular debris.

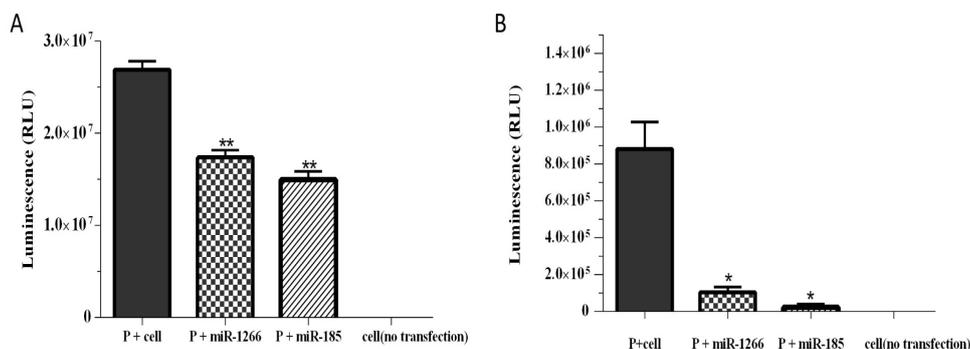


Figure 4. luciferase Assay for Selected microRNAs Targeted to BCL2 3'UTR (A) and BCL2L1 3'UTR (B). P: Plasmid (psicheck 2 vector).

Apoptosis induction

Our results demonstrated that miR-1266-5p and miR-185-5p induced apoptosis in 16.3% and 12.82% of the DU145 cell population, respectively, while the positive control, miR-34a, induced apoptosis in 11.93% of DU145 cells (Figure 3a-d).

In PC3 cells, miR-1266-5p and miR-185-5p induced apoptosis in 7.3 and 9.2% of the cell population compared with miR-34a, which induced apoptosis in 7.3% of the cells (Figure 3e-g).

Identification of BCL2 3'-UTR and BCL2L1 3'UTR as targets of miR-1266-5p and miR-185-5p

The results of the luciferase assay after 8 hrs revealed that both miR-1266-5p and miR-185-5p transfections significantly downregulated Renilla luciferase activity in PC3 cells. These data demonstrated that miR-1266-5p and miR-185-5p regulated BCL2 and BCL2L1 by acting on their 3'-UTRs (Figure 4a and b).

Discussion

Although the goal of cancer treatment is suppressing or eliminating cancer cells without any side effects on the rest of the body, there is no definitive therapy for several types of cancer. Over the past decade, the microRNA pathways have been found to regulate the expression of genes involved in important cancer-generating cellular pathways (Chen et al., 2014). In different studies, downregulation of microRNAs has been identified in various cancer types. The overexpression of certain microRNAs has also been found to be a therapeutic tool in some in vitro as well as in vivo models (Chen et al., 2014; Horsham et al., 2015). On the other hand, miRNAs may modulate chemotherapy in malignancies. It was demonstrated that miR-1266 was associated with poor chemotherapy response in pancreatic cancer patients and upregulation of miR-1266 increased the chemoresistance of pancreatic cancer cells to gemcitabine (Zhang et al., 2018).

Over the last decades, one of the most important methods for prolonging survival and overcoming resistance to treatment in cancer patients is suppressing or silencing the BCL2 gene (Giovannucci, 2001; Yang et al., 2014; Siegel et al., 2015; Tabayoyong and Abouassaly, 2015). Our last study demonstrated the concordance between bioinformatics predictions and/or literature reviews with experimental expression analyses of miR-1266, miR-185 and miR30c, as well as BCL2 and BCL2L1 genes in PCa tissues and cell lines (Ostadrahimi et al., 2018). We revealed that downregulation of miR-1266-5p and miR-185-5p, targeting two anti-apoptotic genes (BCL2 and BCL2L1), may be correlated with the molecular pathogenesis of PCa. Furthermore, it was recently observed that miR-1266 and miR-185 was downregulated in PCa tumor tissues and cell lines. Additionally, downregulation of these miRNAs was correlated with upregulation of the BCL2 and BCL2L1 genes (Ostadrahimi et al., 2018). We hypothesized that the overexpression of microRNAs may induce apoptosis in PCa cell lines by downregulating the anti-apoptotic genes BCL2 and BCL2L1. To examine this hypothesis,

we transfected miR-1266-5p and miR-185-5p into the PC3 and DU145 cell lines. First, the luciferase assay revealed that these two microRNAs bound to the 3'-UTR region of the anti-apoptotic genes, namely BCL2 and BCL2L1. We also observed that introduction of these two microRNAs induced apoptosis in PCa cells. Our findings were supported by those of previous studies evaluating overexpression of miR-1266-5p or miR-185-5p in various cancer tissues.

The role of miR-185-5p in apoptosis induction in various PCa cell lines has been shown previously to be mediated through targeting different genes. Li et al. demonstrated that miR-185-5p targets the Sterol regulatory element-binding protein (SREBP) metabolic pathway, while Qu et al. revealed that the same microRNA targets PCa cells through targeting the androgen receptor (Li et al., 2013; Qu et al., 2013). Wang et al., (2014) also demonstrated that the expression level of miR-185-5p in the samples of breast cancer patients was downregulated in comparison with healthy subjects. The study was also indicated that miR-185 exerted an inhibitory effect on cancer cell proliferation and invasion via targeting the 3'-UTR of the vascular endothelial growth factor (VEGF)-A. Of note, there is a high similarity between the 3'-UTR sequences of VEGF-A and BCL2 mRNAs in the seed region of miR-185-5p (www.microna.org). Imam et al., (2010) also previously reported downregulation of miR-185 in ovarian and breast cancer cell lines. It was also demonstrated that miR-185 bound to the seed region of the Sineoculis homeobox homolog 1 (SIX1) oncogene, which is very similar to the BCL2 mRNA seed region (www.microna.org). Based on our results, it may be hypothesized that up regulation of miR-185-5p may promote apoptosis via multiple off-target interactions.

Apoptosis induction via BCL2 3'-UTR targeting by other microRNAs has been repeatedly demonstrated. Singh et al. revealed that miR-24-2, miR-365-2, and miR-195 targeted BCL2 3'-UTR and led to cell apoptosis (Singh and Saini, 2012). Zhao et al., (2012) demonstrated that miR-125b could bind to the 3'-UTR of the BCL2 gene in hepatocellular carcinoma (HCC) and halted cell proliferation, while promoting cell apoptosis. In 2017, the anti-apoptotic role of miR-340 through targeting BCL2 was revealed in gastric cancer (Yu et al., 2017). In the same study, SGC-7901 was transfected with a miR-340 mimic that promoted cell apoptosis. Yang et al., (2014) . also reported that the miR-16 expression level decreased in brain glioma tissue, whereas its enhancement promoted cell apoptosis via BCL2 suppression, and eventually suppressed tumor growth and invasion.

In 2015, suppression of BCL2L1 expression was identified in B-cell lymphoid cells following overexpression of miR-377, which was correlated with an increase of apoptotic cell death. Binding of miR-377 to BCL2L1 3'-UTR was revealed by bioinformatics and was subsequently established by a luciferase assay (Al-Harbi et al., 2015).

In our previous study, we confirmed the downregulation of miR-1266 and miR-185, and the upregulation of the BCL2 and BCL2L1 genes in PCa tissues and cell lines. Moreover, the results of the present study confirmed the

miR-1266 and miR-185 to act as tumor suppressors in PCa cell lines by inducing cell apoptosis and decreasing proliferation via inhibiting the BCL2 and BCL2L1 genes. Since the effects of microRNAs on target genes and protein translation is known, it is required to initially determine the effects of miR-1266 and miR-185 on protein translation by tests, such as western blotting. Further investigations are warranted to confirm downregulation of BCL2 and BCL2L1 expression following treatment with microRNAs, and using in vivo and ex vivo models should also be considered for further investigations.

Disclosure of conflict of interest

The authors declare there are no conflicts of interest with regard to the publication of the present study.

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