Pakistan J. Zool., vol. 52(5), pp 1675-1682, 2020 DOI: https://dx.doi.org/10.17582/journal.pjz/20190603070617

MicroRNA-16 Down-regulates BCL2 Expression and Induces Papillary Thyroid Carcinoma Cell Apoptosis Via Extracellular-regulated Kinase Pathway

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ABSTRACT

Papillary thyroid cancer is one of the most common tumours subtype in thyroid cancers. Substantial amount of data have indicated that miR-16 is an important regulator of various tumours; however, the molecular functions of miR-16 in papillary thyroid cancer remained largely elusive. In order to investigate the regulation of miR-16, human papillary thyroid carcinoma cells are transfected with miR-16 mimics or inhibitors. Targeting miR-16 and using dual-luciferase reporter assay, qRT-PCR and Western blot, the mRNA and protein expression were detected, respectively. Cellular apoptosis were examined by flow cytometry and Western blots was applied to assess the cleaved caspase-3, -8, -9, and cleaved poly-ADP-ribose polymerase. Protein expression of extracellular-regulated kinase were detected. Finally, data analysed statistically to determine significantly differences among groups. Results showed that breakpoint cluster 2 was a direct target of miR-16, and the expression was negatively correlated. Moreover, miR-16 promoted apoptosis suppressing breakpoint cluster 2 through phosphorylation of extracellular-regulated kinase. The study validates miR-16 as a tumour suppressor gene in papillary thyroid cancer cells, and revealed a novel mechanism of miR-16-mediated apoptosis through extracellular-regulated kinase pathway.

Article Information
Received 03 June 2019
Revised 30 July 2019
Accepted 15 October 2019
Available online 06 May 2020

Key words
Papillary thyroid carcinoma, miR-16, Extracellular-regulated kinase, Apoptosis, BCL2

INTRODUCTION

During past several decades, increasing incidences of papillary thyroid carcinoma (PTC) have been observed in several countries around the globe (Baser *et al*., 2015). In China, the incidence rate of thyroid cancer is rising by approximately 14.5% per year (Alath *et al*., 2015). After confirmed diagnosis of PTC, the conventional treatment is surgical excision (Agosto-Vargas *et al*., 2017). However, surgeries pose a high rate of postoperative lymph node recurrence or metastasis (5% to 40%), because of its biological characteristics of easy invasion of the surrounding lymph nodes (Ahn *et al*., 2015). Besides, the long-term follow-up monitoring in thyroid cancer patients, continues ultrasound and serum thyroglobulin (HTG) monitoring is the most common detection indices and yet the indices of PTC recurrence are significantly increasing (Al-Qahtani *et al*., 2015). Treatment of the patients with I131 radiotherapy alone was effective for limited cohorts with recurrent and metastatic lesions (Anajar *et al*., 2017). Nevertheless, up to 30% of PTC is not sensitive to I131 therapies, which sets obvious limitation on the therapeutic implication of I131 radiation therapy (Angell, 2017). American Thyroid Association guidelines indicate that surgery is the optimal treatment for the recurrence of PTC, which has given perfect performance (Asa and Ezzat, 2017). However, due to the damaged anatomical structure and a large amount of fibrous and scar tissues in the surgical field (Bingol *et al*., 2015), re-operation of papillary thyroid carcinoma is cumbersome and risky (Atespare *et al*., 2015). In order to explore better therapeutic strategies to cure PTC, a higher resolution understanding on the biological mechanism involved in PTC is becoming increasingly important.

MicroRNAs (miRNAs) are involved in the pathogenesis of diverse human cancers including PTC through their regulatory affects on the target genes (Zheng *et al*., 2016). However, there are few studies regarding associations between clinic-pathological features of PTC with the expression of specific miRNAs and their potential target genes (Cong *et al*., 2015). The miRNAs, a class of non-coding RNA (Shahid *et al*., 2019), can act as both tumour suppressors and oncogenes by negatively regulating their mRNA targets through degradation or translational repression (Cheng *et al*., 2017).

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0030-9923/2020/0005-1675 $ 9.00/0

Abbreviations
PTC, Papillary thyroid cancer; BCL2, breakpoint cluster 2; PARP, poly ADP-ribose polymerase; HTG, thyroglobulin; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK,mitogen-activated protein kinase; MEK, MAPK or ERK Kinase; ATCC, American type culture collection; DMEM, Dulbecco's Modified Eagle medium.

Previous studies have indicated that down-regulation of miR-16 in a wide range of cancers, including breast, prostate and lung cancers (Gu *et al*., 2015), as well as in chronic lymphocytic leukemia (Fennell, 2017). These findings suggest that miR-16 is a possible tumor suppressor that acts in a variety of cancers. It has also been proposed that the expressions of miR-16 in PTC tissues might be a useful biomarker and promising targets in the diagnosis of PTC (Hu *et al*., 2017). However, the molecular mechanisms of miR-16-mediated regulation of PTC remained largely unknown.

The cell intrinsic apoptosis pathway is regulated by breakpoint cluster 2 (BCL2) proteins, which control the release of cytochrome c from the mitochondria, triggering the activation of caspases and cell death (Moschos *et al*., 2017). In addition, independent studies have uncovered new mechanisms by which the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway can control the activity or abundance of members of the BCL2 protein family to promote cell survival (Kwon *et al*., 2017). There are abundant evidences that survival factors can use the ERK1/2 pathway to increase the expression of several pro-survival BCL2 proteins, notably BCL2, by promoting de novo gene expression in a variety of cell types (Pillai *et al*., 2013). For example, mitogen-activated protein kinase (MAPK) or ERK Kinase (MEK) inhibition lead to a decrease in BCL2 and apoptosis in pancreatic cancer cells (Qin *et al*., 2014). It is now apparent that various oncogenes co-opt ERK1/2 signalling to de-regulate BCL2 proteins and this contributes to, and even underpins, survival signalling in multiple tumours (Radha and Raghavan, 2017). New oncogene-targeted therapies allow direct or indirect inhibition of ERK1/2 signalling and can cause striking tumour cell death (Rossi *et al*., 2008). In other cases, inhibition of the ERK1/2 pathway may be highly effective in combination with other conventional and novel therapeutics (Akiyama *et al*., 2014). Nevertheless, it is unknown whether PTC cell lines may undergo apoptosis upon inhibition of the ERK1/2 pathway and such understanding can facilitate the development of novel therapeutics in future.

Finding of this study revealed that miR-16 can target BCL2 gene and investigated the correlation between these two biomolecules. In additions, the study revealed a novel mechanism of miR-16-mediated facilitation of apoptosis by inhibiting BCL2 expression through ERK pathway. Taken together, the study has laid foundation for future in-depth investigations on the biological effect of miR-16 in human PTC cells.

MATERIALS AND METHODS

Cell cultures and oligonucleotide transfection

The human papillary thyroid carcinoma cells (PTC-1) and 293T cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C in 5% CO_2. For miR-16 over-expression, cells were transfected with 100 nmol/L of miR-16 mimics. The miR-16 mimics were small, and chemically modified double-strand RNA molecules that were designed to mimic endogenous mature miRNAs. For inhibition, cells were transfected with miR-16 inhibitors, which were chemically modified, single-strand oligonucleotides designed to specifically bind to and inhibit endogenous miRNAs.

The sequences were as follows: for miR-16 mimics: 5'-UAGCAGCACGUAAAUAUUGGCGCCAAU-AUUUACGUGCUGCUAUU-3'; for the negative control oligonucleotide, UGUCACGUTTACGUGA-CACGUUCGGAGAATT-3'; for miR-16 inhibitors, 5'-CGCCAAUAUUUACGUGCUGCUA-3'; and for the negative control oligonucleotide, 5'-CAGUACUUUU-GUGUAGUACAA-3'.

Cells were seeded in 6-well plates and upon reaching to 70-80% confluency, these were transfectioned using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. To achieve stable cell lines that overexpressed miR-16, 293T cells were co-transfected with the pcDNA6.2-GW/EmGFP-miR plasmid that contains computer-designed oligonucleotide sequences expressing the pre-miR-16. The medium was replaced 4h post-transfection and the cells were incubated in a cell incubator.

Dual-luciferase reporter gene assay

The 3'-UTR of BCL2 gene, which contains one putative miR-16 targeting site, was chemically synthesed and inserted into the XhoI/SacI and SacI/HindIII sites of the pMIR-REPORT vector (Ambion, Austin, TX, USA). To express miR-16, a genomic fragment encompassing the coding region was cloned by PCR and inserted into the XhoI and KpnI sites of pCMV vector. In total, 40000 cells were seeded in 24-well plates 24h prior to transfection. Cells were co-transfected with 0.1ug pMIR-BCL2, or pMIR-REPORT, together with 40nM miR-16 precursor molecule or 40nM negative control. The pRL-TK vector (Promega, Madison, WI, USA) containing Renilla luciferase was also co-transfected as a reference control. Firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay (Promega) 24h after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity.

Quantitative RT-PCR analysis of mRNA and miRNA expression

Total RNA from transfected cells was isolated

using TRIzol reagent (Invitrogen) for both mRNA and miRNA analyses. Relative levels of BCL2 mRNA were examined using SYBR green real-time quantitative RT-PCR (qRT-PCR) (Light Cycler 480 Roche, Switzerland) and were normalized to levels of housekeeping GAPDH mRNA. Following primers were used: BCL2 forward, 5'-ATCGCTCTGTGGATGACTGAGTAC-3'; BCL2 reverse, 5'-AGAGACAGCCAGGAGAAATCAAAC-3'; GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3'; GAPDH reverse, 5'-AGGGGCCATCCACAGTCTTC-3'. For analysis of miR-16 expression, qRT-PCR was carried out using the All-in-One miRNA qRT-PCR Detection Kit (Gene Copoeia, Rockville, MD, USA) according to the manufacturer's instructions (Light Cycler 480 Roche) and was normalized to the expression of U6 (miR-16 primer ID, hsmq-0031, Catalog# HmiRQP0227; U6 primer ID, hsRNAU6, Catalog#HmiRQP9.001; Light Cycler 480 Roche). Relative expression was calculated using the $2^{-\triangle\triangle CT}$ method. All qRT-PCR analyses were carried out in triplicate, and the data were presented as means±standard errors of the means.

Cell apoptosis assay

To analyse the rates of cell apoptosis, a commercially available annexin V-FITC kit was used (BD Biosciences, Franklin Lakes, NJ, USA). After 24 h of treatment, cells were harvested by trypsinisation, collected by centrifugation, and washed twice with cold PBS. Each sample of cells was then re-suspended in 200 µL 1×binding buffer and subsequently incubated with 2 µL annexin V-FITC and 2µL propidium iodide (PI). After an incubation period in the dark at room temperature for 15 min, the population of apoptotic cells in each cell sample was detected by flow cytometry within 1 h of completing the incubation period. All analyses were carried out in triplicate.

Western blot analysis

After the treatment with miR-16, the cell lysates of PTC were collected and the concentration of the total protein was determined using the Protein Assay kit (Bio-Rad, Hercules, CA, U.S.A.). Cell extracts with sample buffer were boiled for 5 min and then separated by 10% SDS–PAGE gel. After electrophoresis, the gel was transferred onto a PVDF membrane for immunoblotting The membrane was first blocked by incubation in non-fat milk at room temperature for 2h, then incubated with anti-phospho-ERK1/2 (p185/187) antibodies (BioSource International Inc., Camarillo, CA, U.S.A.), anti-ERK1/2, anti-Bcl-2 antibodies (Upstate Biotechnology, Waltham, MA, U.S.A.), or anti-actin as loading control (Chemicon International, Temecula, CA, U.S.A.), caspase-3 (Danvers, MA, USA), PARP (Danvers, MA, USA), caspase-8 (Danvers, MA, USA), or caspase-9 (Danvers, MA, USA) for 2h at room temperature. Membranes were washed for five times with Tris-Buffered Saline Tween-20, and then incubated further at room temperature with horseradish peroxidase-conjugated secondary antibodies for 2h. Then, the membrane was washed six times by TBST and specific bands were visualized by chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Statistical analysis

The numerical data were presented as mean ± SD. Two-tailed Student's t-tests are used to analyse the differences between two groups. One-way analysis of variance (ANOVA) was performed for analysis of multiple groups. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 21.0 software (SPSS, Chicago, IL, USA).

RESULTS

miR-16 directly targets BCL2 and expression of miR-16 and BCL2 is negatively correlated

As shown in Figure 1A, the microRNA database shows that BCL2 is a potential target for the miR-16-mediated post-transcriptional repression. A dual-luciferase reporter assay system was used to validate whether miR-16 directly recognizes the 3'-UTR region of BCL2 mRNA. The relative luciferase activities of the pre-miR-16 transfected with BCL2 3'-UTR constructs were significantly decreased (Fig. 1B), compared to those transfected with pMIR-BCL2 constructs in 293T cell, implying that BCL2 is a direct target of miR-16.

To further demonstrate the correlation between miR-16 and BCL2, mRNA expression of BCL2 and miRNA expression of miR-16 were detected by qRT-PCR. As shown in Figure 1C, miR-16 up-regulation dramatically reduces the expression of BCL2 gene. Therefore, the miR-16 expression is inversely correlated with the BCL2 expression in PTC cells.

In order to further investigate the influence of miR-16 mimics and inhibitors on inducing apoptosis in PTC cells, BCL2 proteins were detected using Western blot in various conditions. As shown in Figure 1D, over-expression of miR-16 led to substantial decrease in the BCL2 expression further supporting the negative regulatory effect of miR-16 on the expression of BCL2.

miR-16 promotes apoptosis

Cleavage of caspase-8, caspase-9, caspase-3, and poly-ADP-ribose polymerase (PARP) to their active forms are very important events in cancer cell apoptosis (Mondal *et al*., 2016). Therefore, levels of these apoptosis-related proteins were assessed using Western blot.

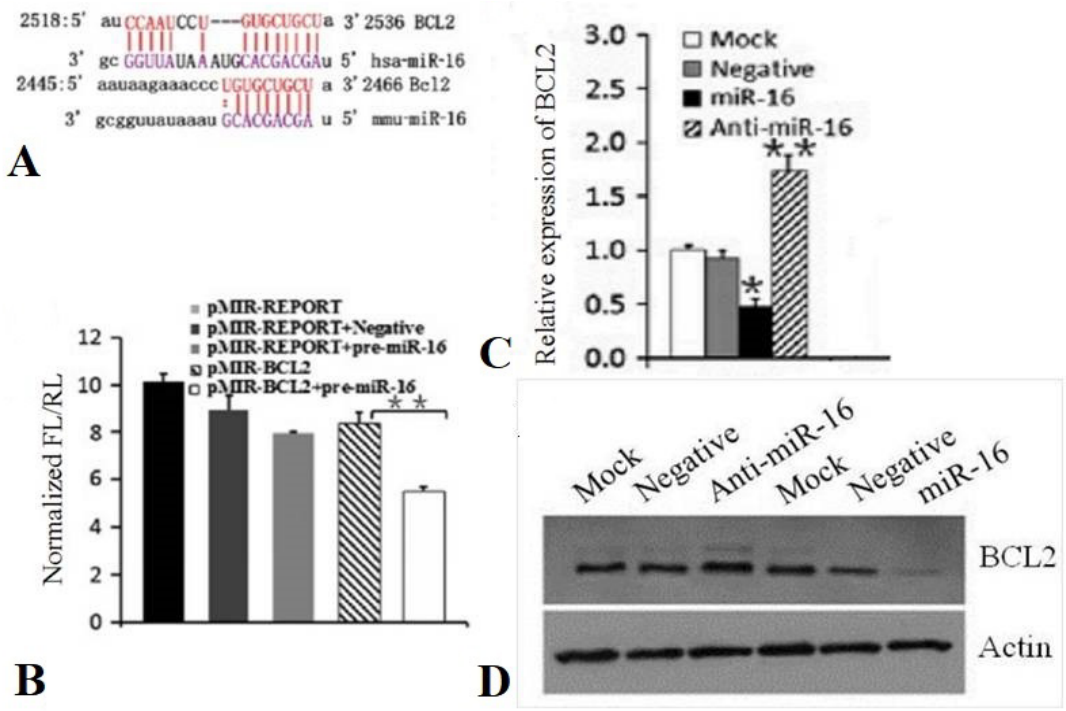

Fig. 1. miR-16 directly targets BCL2 and decrease BCL2 expression. (A) analyzing the homology between miR-16 and BCL2 mRNA sequences. (B) Luciferase assay revealed reduced relative luciferase activities in 293T cells stably over-expressing miR-16 following transfection of BCL2 3'-UTR using pMIR and pMIR-REPORT vectors ($P<0.01$). FL, firefly luminescence; RL, Renilla luminescence. (C) Quantitative RT-PCR analysis showed that miR-16 inhibited the expression of BCL2. *$P < 0.05$; **$P < 0.01$. (D) Western blot analysis showed that miR-16 led to obvious decrease of BCL2 expression.

As shown in Figure 2A, levels of cleaved caspase-8, caspase-9, caspase-3, and PARP increased following treatment with miR-16, compared with negative group.

Additionally, PTC cells transfected with miR-16 mimics showed a larger number of G0/G1 stage of cell division than the negative control, whereas cells in the G2 or S stages showed no significant variations (Fig. 2B). These findings mean that miR-16 arrests PTC cells at G0/G1 stage and promotes apoptosis.

To further delineate molecular mechanisms involved, the percentage of cells undergoing apoptosis following the various treatments was detected by flow cytometry. As shown in Figure 2C, a higher percentage of cells undergoing apoptosis was observed for miR-16 group as compared with negative control. Quantitative analysis of results showed corresponding differences and confirmed finding of the flow cytometry (Fig. 2D). Taken together, these results indicate that miR-16 promotes apoptosis in PTC cells.

miR-16 decreases BCL2 expression through phosphorylation of ERK1/2

To determine the biomolecular mechanism of the miR-16-induced BCL2 expression, the protein signal transduction pathway was further explored. As shown in Figure 3A, cells with no miR-16 and no PD98059 treatment, the expression level of BCL2 in control group carried no obvious changes as the time increased. However, as shown in Figure 3B, the cells treated with miR-16 but not with PD98059, miR-16 decreased the BCL2 expression for 3 and 5 days. Concurrently, a lower expression level of phosphorylated ERK 1/2 was detected. PD98059, an MEK inhibitor, was applied in order to evaluate whether the activated ERK1/2 proteins are associated with BCL2 expression in the miR-16 treated PTC cells. As shown in Figure 3C, cells treated with miR-16 and PD98059, expression of BCL2 and phosphorylated ERK1/2 with miR-16 treatment were significantly inhibited by PD98059. In contrast, there were no significant changes in total amounts of ERK proteins. Therefore, these finding indicate that miR-16 decreased the BCL2 expression through the ERK pathway.

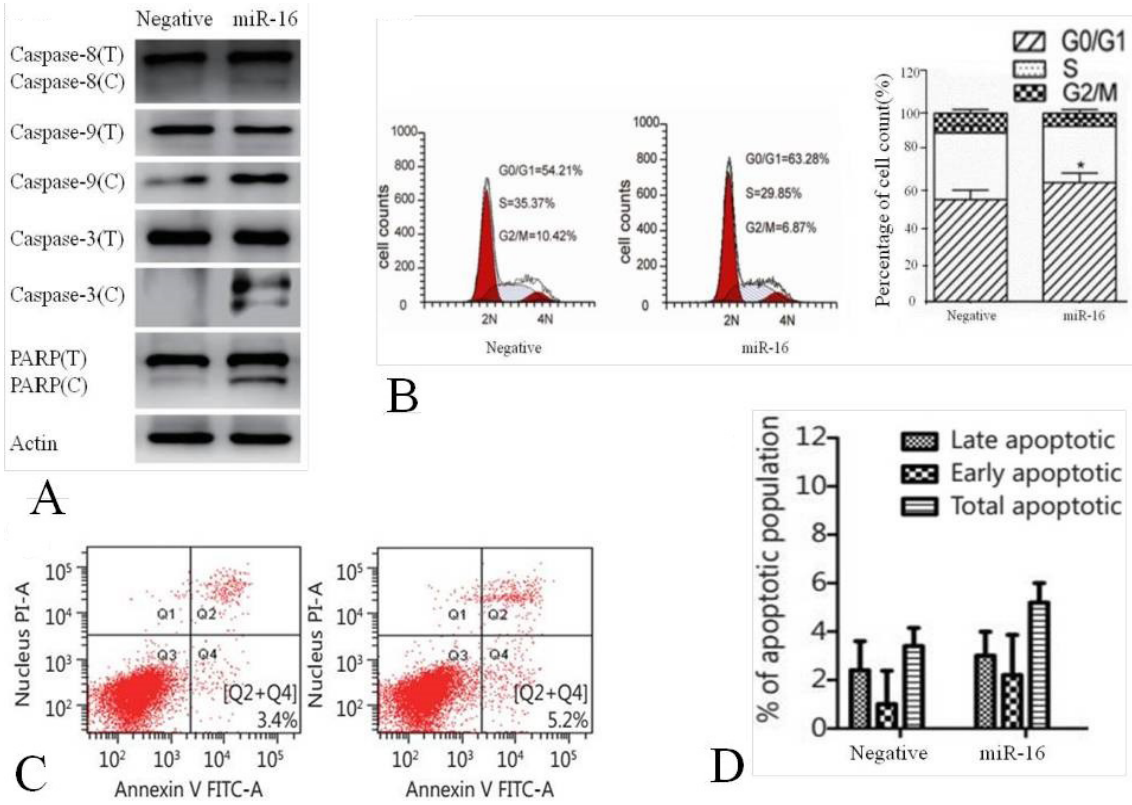

Fig. 2. miR-16 increase apoptosis. (A) Lysates from PTC cells treated with miR-16 for 24 h were analyzed. Levels of cleaved caspase-8, caspase-9, caspase-3, and PARP were detected and compared with the total levels of each protein (labeled with C vs. T). Detection of Actin was performed as a loading control. (B) PTC cell cycle was analyzed by flow cytometer (PI staining) 48 h post transfection. In the group of miR-16, the proportion of cells at G0/G1 stage was much higher than negative groups ($P<0.05$). (C) Apoptosis was detected in the negative and miR-16 group of PTC cells by flow cytometry. (D) Quantification of the flow cytometry data according to apoptosis stage is shown with the error bars representing standard deviation values. $P < 0.01$, based on the Student's t-test.

DISCUSSION

Previous studies indicate that miRNAs are important factors in the pathogenesis of human PTC (Jiang *et al*., 2014). Pallante *et al* have reported that miR-221, -222 and -181b were up-regulated in human PTCs (Pallante and Pich, 2000). Similarly, Zhang *et al*. (2017) have reported that miR-21 was over-expressed in human PTCs, decreasing the expression of miR-21 could inhibit the human PTC cells proliferation, invasion and induced the apoptosis through the regulation of programmed cell death. Additional studies have reported that miR-221, -222, and -146 were top most up-regulated microRNAs in human PTC whereas miR-219, -138, -345 and -26a were down-regulated in human PTCs (He and Huang (2017)). Despite of these clear involvements of microRNA in cancer regulation, the biological effects of miR-16 on human PTC and other tumors are not defined. In the present study, it has been demonstrated that miR-16 is a tumor suppressor gene that induces PTC cells apoptosis. Moreover, the key finding of this study was that miR-16 levels were inversely correlated with BCL2 expression. Additionally, a novel mechanism of miR-16-mediated regulation in PTC cells apoptosis through inhibition of BCL2 and ERK pathway was revealed.

In this study, the biological effect of miR-16 in human PTC cells was investigated. The BCL2 is an oncogene (Anderson *et al*., 2015), which controls the release of cytochrome c from mitochondria, triggering the activation of caspases and cell death (Ahmed *et al*., 2014). In this study, it was found that miR-16 directly targets BCL2 and induces PTC cells apoptosis through inhibition of BCL2 and ERK pathways. Clearly, the ERK1/2 pathway can regulate several members of the BCL-2 protein

family to achieve cell survival (Bluthgen *et al*., 2017). However, many of these proteins are also controlled by other oncogene-regulated signalling pathways (Calvo *et al*., 2017). In additions, the BCL-2 protein family is recruited to initiate death when the ERK1/2 pathway is inhibited (Casar and Crespo, 2016), suggesting that inhibitors of the ERK1/2 pathway may show potential in teatign cancers independently or in combination with other therapies (Chen *et al*., 2016). However, it is still unclear how ERK1/2 pathway regulates BCL2 in human PTC and warrants future studies.

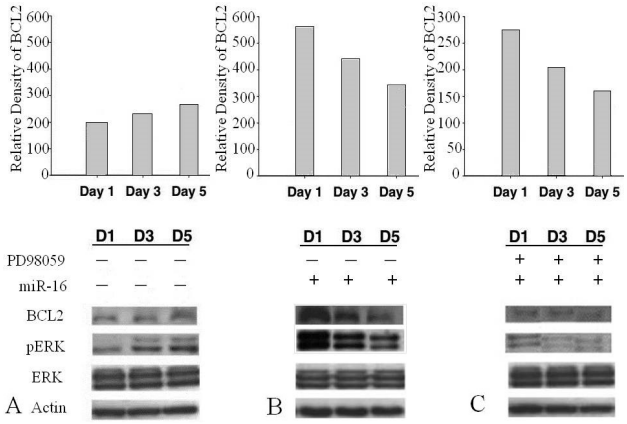

Fig. 3. miR-16 decreases BCL2 expression through ERK1/2 pathway. (A) Detection of the protein amount of BCL2, phosphorylated ERK1/2, and total of ERK1/2 in day-1, day-3 and day-5 PTC cells with miR-16 treatment by western blotting assay. The cells with no miR-16 and no PD98059 treatment. The expression level of BCL2 in control group have no obvious change with the time increased. (B) The cells treat with miR-16 but no PD98059, miR-16 decrease BCL2 expression for 3 days and 5 days. Concurrently, a lower expression level of phosphorylated ERK 1/2 is detected. (C) The cells treat with miR-16 and PD98059, the expressions of BCL2 and phosphorylated ERK1/2 are significantly inhibited by PD98059. In contrast, there is no significant change in the total amounts of ERK. $P < 0.01$, based on the Student's t-test.

The sensitivity and specificity of biomarkers, which have been used in clinical practices for diagnosis of PTC, are low. Therefore, it is essential to develop novel diagnostic and prognostic biomarkers for PTC (Zhu *et al*., 2016). Some studies have shown that expressions of miR-16 in PTC tissues might be useful biomarkers and promising targets in the diagnosis of PTC (Guo *et al*., 2016). Other studies have demonstrated miR-16 may be tumour invasion suppressors in PTC (Rissland *et al*., 2011). Moreover, it also indicates that miR-16 is involved in the tumour stem cell apoptosis process and is down-regulated in the invasive subpopulations compared with the control subpopulations as shown by Gao *et al* in human PTC cell lines (Gao *et al*., 2014). Combination with this study, it suggests that miR-16 may be a novel biomarker and therapeutic target in PTC.

The accumulating reports proposed an appealing concept that sequence-specific inhibition of miRNAs in stem/progenitor cell populations can deliver a potential therapeutic strategy for modulation of stem/progenitor cells whose miRNAs are de-regulated in cancers (Zhu *et al*., 2015). Previous studies show that miR-16 is a possible tumour suppressor that acts in a variety of cancers (Usmani *et al*., 2017). In additions, Yang *et al* have reported that miR-16 is a tumor suppressor gene in glioma growth and invasiveness, and their finding suggest the possible future use of miR-16 as a therapeutics in gliomas (Yang *et al*., 2017). Taken together, this study proposes appropriate application of miR-16 as a novel avenue for the treatment of human cancers in the future.

CONCLUSION

The study revealed that miR-16 targets and negatively regulate BCL2 gene. In additions, miR-16 promotes apoptosis by inhibiting BCL2 through ERK pathway. Clearly, understanding the molecular basis involved in the development and aggravation of PTC will be helpful in identifying novel diagnostic, prognostic and therapeutic targets. Although the detailed mechanisms and clinical applications of miR-16 are yet to be fully elucidated, miR-16 expression in PTC not only provides a unique supplemental tool for diagnosis and predicting prognosis, it may also serve as a novel biomarker and therapeutic target for PTC in the near future.

Statement of conflict of interest

Authors have declared no conflict of interest.

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