miR-204 acts as a tumor suppressor in human bladder cancer cell T24 by targeting antiapoptotic BCL2

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Objective: Bladder cancer (BC) is one of the most common noncutaneous malignant cancers with high mortality and recurrence rates in the general population, particularly in men. We previously reported detecting dysregulated micro-RNAs (miRNAs) in human BC tissues. Using an miRNA targeting database, we found that miR-204, which is downregulated in BC, targets the B-cell lymphoma 2 gene (BCL2). We hypothesized that the overexpression of miR-204 induces apoptosis by repressing BCL2 expression and causing cancer cell death.

Materials and methods: To overexpress miR-204 in cancer cells, a vector that expresses miR-204 was transfected into human BC, embryonic kidney, and cervical cancer cells. The expression of miR-204 in the transfected and mock cells was monitored through miRNA quantitative polymerase chain reactions (Q-PCRs). Luciferase reporter assays were performed to verify the direct binding of miR-204 to targeted sites on the BCL2 transcripts. The BCL2 mRNA and protein expression levels in the miR-204-transfected cells were measured using Q-PCRs and Western blotting, respectively. The cell viability of the miR-204 transfected cells was detected through WST-1 assays. The induction of apoptosis was determined by the increased release of cytochrome c, cleavage caspase 3, caspase 3/7 activity, and DNA fragmentation.

Results: The expression of miR-204 in the transfected cells was elevated to 6.2-fold, compared with the controls, and the function of matured miR-204 was confirmed through the luciferase reporter assays. The expressions of BCL2 mRNA and protein were reduced in the miR-204-transfected cells, which generated increased DNA fragmentation, caspase 3 cleavage, caspase 3/7 activity, and reduced cell viability. Cotransfection of the reporter vector that harbored the BCL2 3′-untranslated region to compete with endogenous transcripts partially rescued the apoptosis that was induced by the overexpressed miR-204. This finding further supported that miR-204 targeting BCL2 induces apoptosis in BC cells.

Conclusion: miR-204 targets BCL2 in human BC cells. The overexpression of miR-204 in tumors could thus be a novel strategy for inducing cell death by targeting antiapoptotic BCL2.

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1. Introduction

In developed countries, bladder cancer (BC) is the fifth most diagnosed tumor and the second leading cause of death in patients with genitourinary tract malignancies. In the United States, more than 73,000 new cases and 14,000 deaths occur annually. In 2011, BC was the ninth leading cause of cancer-related deaths in men in Taiwan. BC remains one of the most costly cancers with regard to treatment and the monitoring of cytological changes through approaches such as surveillance cystoscopy and periodic imaging. Despite the existence of appropriate therapies, patients with BC are continuously at risk of its recurrence and progression to the muscles. Therefore, developing new treatment strategies to reduce these risks by utilizing novel molecular networks is highly desirable.

Recent studies have shown that micro-RNAs (miRNAs), which are key post-transcriptional regulators, act as tumor suppressors or
oncogenes in cancer progression. Furthermore, since the discovery of miRNAs, a growing number of studies have reported that dysregulated miRNAs are common in human cancer and are involved in various physiological and pathological processes. A previous study compared patients’ BC tissue with their adjacent normal bladder tissue and found dysregulated miRNAs, including 19 upregulated and 11 downregulated miRNAs, in the BC tissue. These downregulated miRNAs may act as tumor suppressors; therefore, their decreased expression promotes cancer cell survival. In silico approaches revealed that miR-204, which is downregulated in BC, potentially targets the B-cell lymphoma 2 gene (BCL2), an antiapoptotic protein. In this paper, we showed that BCL2 expression is regulated by miR-204, which directly interacts with the 3′-untranslated region (3′-UTR) targeting site.

2. Materials and methods

2.1. Construction of the vectors

To construct the miR-204 expression vector, a paired oligonucleotide consisting of matured miR-204 sequences was synthesized and cloned into a small-RNA expression vector, pSM, as described in Materials and methods. Negative controls, 5 and designated them as pSM-15a and pSM-214, were constructed by exposure to a polymer-based transfection reagent (Transfec 293; GeneDireX, Gueishan Township, Taiwan) for the luciferase assay, double strand oligonucleotides bearing 19 bases upstream and 11 bases downstream of the miR-204 or miR-15a seed sequences of the BCL2 3′-UTRs were cloned into the pmirGLO vector and designated as pmiR-Bcl2-204TS and pmiR-Bcl2-15TS, respectively. The vectors containing an MTS (i.e., pmiR-Bcl2-204TS and pmiR-Bcl2-15MTS) were constructed through a similar cloning step by using the reverse seed sequences of miR-204 and miR-15a, and these vectors served as the negative controls. The oligonucleotides are listed in Table 1.

To test whether pSM-204 or pSM-15a generates matured and functional miRNAs, we constructed reporter vectors (pmiRGO; Promega, Madison, WI, USA) with antisense [i.e., positive targeting site, (PTS)] sequences or sense [i.e., mutated targeting site (MTS)] sequences of miR-204 and miR-15a, respectively. This cloning step generated pmiR-204PTS, pmiR-204MTS, pmiR-15aPTS, and pmiR-15aMTS. To construct reporter plasmids containing an miR-204 targeting site in the 3′-UTR of BLC2 for the luciferase assay, double strand oligonucleotides bearing 19 bases upstream and 11 bases downstream of the miR-204 or miR-15a seed sequences of the BLC2 3′-UTRs were cloned into the pmirGLO vector and designated as pmiR-Bcl2-204TS and pmiR-Bcl2-15TS, respectively. The vectors containing an MTS (i.e., pmiR-Bcl2-204MTS and pmiR-Bcl2-15MTS) were constructed through a similar cloning step by using the reverse seed sequences of miR-204 and miR-15a, and these vectors served as the negative controls. The oligonucleotides are listed in Table 1.

2.2. Cell culture and transfection

Human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37°C under 5% carbon dioxide. The human BC cell line T24 (HTB-4) was cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen Carlsbad, CA, USA). The human embryonic kidney cell line T293 (CRL-11268) cell line and cervical cancer HeLa (CCL-2) cell line were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen). The media were supplemented with 10% fetal bovine serum (Invitrogen), 2mM GlutaMAX-I (Invitrogen), and 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Cells were transfected using miRNA-expressing vectors with or without reporter constructs by exposure to a polymer-based transfection reagent (Transfec 293; GeneDireX, Gueishan Township, Taiwan) for quantitative polymerase chain reaction.

Table 1

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<tr>
<th>Name</th>
<th>Sequences (5′ → 3′)</th>
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Bot — bottom; EGFP — enhanced green fluorescent protein; miRNA — micro-RNA; Q-PCR — quantitative polymerase chain reaction.
24 hours. In some samples, 400 μg/mL G418 was added to the medium for 24 hours before the cells were harvested.

### 2.3. Real-time quantitative polymerase chain reaction

The NCode miRNA First-strand cDNA Synthesis Kit (Invitrogen) and NCode SYBR Green miRNA qRT-PCR Kit (Invitrogen) were used to detect the expression of miR-204 in the transfected cells, as described in a previous study. To normalize the relative abundance of miRNAs, U6 RNA was employed as an internal control. The expression of BCL2, MCL-1, and BAD was measured in the 24-hour post-transfected cells. The total RNA was isolated using a TRIzol reagent (Invitrogen). The RNA quality and yield were measured using a spectrophotometer (Nanodrop 2000; Thermo Scientific, Waltham, MA, USA). Reverse transcription and quantitative polymerase chain reaction (Q-PCR) were performed, as described previously. Quantification was conducted in triplicate, and the data were derived from three independent experiments. Enhanced green fluorescent protein (EGFP) coexpressed with miR-204 was used as an internal standard to eliminate potential differences in transfection efficiency. Data were collected from triplicate results from different batches of RNA samples. These data are expressed as the mean ± the standard deviation (SD). The specific primer pairs used in this study are listed in Table 1.

### 2.4. Luciferase reporter assays

The 293T cells were cotransfected with pSM-204, miR-15a, pSM-15 or a control (i.e., pSM), and reporter plasmids. Firefly and Renilla luciferase activity were measured consecutively at 24 hours post-transfection in accordance with the manufacturer’s instructions in the Dual-Luciferase Kit (Promega). The relative protein levels were expressed as Firefly–Renilla luciferase ratios. The data were retrieved from triplicate wells through three independent experiments, and are expressed as the mean ± SD.

### 2.5. Detection of BCL2 protein levels through Western blotting

At 24 hours post-transfection, the medium was replaced with a complete medium containing G418 (400 μg/mL). Protein samples were collected at 96 hours post-transfection using a radioimmunoprecipitation assay (RIPA) buffer (150mM sodium chloride, 10mM Tris, pH 7.2, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100, 5mM EDTA, pH 8.0) containing a 1× protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Waltham, MA, USA). Western blotting was subsequently performed, as described in a previous study. Band signals were acquired in the linear range of the scanner using densitometry software (TotalLab Quant V1.0.8, TotalLab Ltd., Newcastle upon Tyne, UK). The ratio between the BCL2 and the EGFP bands was used to quantitate BCL2 modulation by miR-204.

### 2.6. Cell viability and detection of apoptosis

For cell viability assays, the cells were transfected with pSM-204 with or without reporter vectors, as described in section 2.2. Cell viability was measured at 24 hours post-transfection using a WST-1 reagent (Roche). We detected the release of cytochrome c from the mitochondria to the cytosol and the presence of caspase 3/7 activity in the transfected cells for apoptosis assessment. The cytosol protein fractions in the transfected cells were isolated using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) in accordance with the manufacturer’s instructions, electrophoresed on 15% polyacrylamide gel, and then analyzed through Western blotting using an anticytochrome c antibody (#136F3; Cell Signaling Technology, Beverly, MA, USA), voltage-dependent anion channel [(VDAC); #D73D12; Cell Signaling Technology], and enhanced chemiluminescence, as described previously. The caspase 3/7 activity was assayed, as indicated in a previous study. The measured fluorescence was normalized to the fluorescence of the nontransfected cell lysates, and the caspase 3/7 activities were normalized to the cell viability.

### 2.7. Statistical analysis

Data are presented as the mean ± SD of the results of three independent experiments, each of which was performed in triplicate. The Student t test was employed to identify significant differences between the experimental groups. A value of p < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Elevated level of miR-204 in pSM-204 transfected cells

To study the effect of miR-204 on the expression of putative targets in cancer cells, we elevated the expression of miR-204 in BC (i.e., T24) cells. Embryonic kidney (i.e., 293T) cells and cervical cancer (i.e., HeLa) cells were the reference cancer cells. As anticipated, miR-204 expression was higher in pSM-204-transfected cells than in the controls. As displayed in Figure 1, miR-204 transcripts were elevated 3.92 ± 0.42, 6.21 ± 0.24, and 5.67 ± 0.31 times in the T24, 293T, and HeLa cells, respectively.

#### 3.2. miR-204 interacts directly with the predicted targeting site on BCL2

To identify putative miR-204 targets that may contribute to tumorigenesis, we employed an in silico strategy using miRNA target databases such as PicTar and TargetScan. The well-known antiapoptotic BCL2 gene was identified as a putative miR-204 targeted gene. The prediction results and TargetScan score are listed in Figure 2A. The BCL2-like 2 (BCL2L2) gene is an miR-204 targeted gene. We thus included the predicted miR-204/BCL2L2 results as a

![Figure 1](image.png)  
**Figure 1.** Elevated miR-204 expression in T24, 293T, and HeLa cells transfected with pSM-204. Cells were harvested 24 hours post-transfection while coexpressed EGFP was detected under fluorescent microscopy. Total RNA was extracted, reverse transcribed, and followed by an miRNA Q-PCR. The expression of miR-204 was normalized using U6 RNA. The data represent three independent experiments and are expressed as the mean percentage ± the standard deviation, relative to the vector-only control (i.e., pSM); EGFP = enhanced green fluorescent protein; miRNA = micro-RNA; Q-PCR = quantitative polymerase chain reaction. *p < 0.05.
Figure 2. Effect of the putative miR-204 targeting site derived from the BCL2 3′-UTR on luciferase expression. (A) The putative binding sites of miR-204 in the BCL2 and BCL2L2 3′-UTR regions, as detected using the TargetScan database. (B) The BCL2 3′-UTR sequences that contain the putative miR-204 targeting site are highly conserved among vertebrate species. The figure is derived from the TargetScan results, and the miR-204 seed sequences are indicated with a white background. (C) The predicted RNA duplex structure of miR-204/BCL2 and miR-204/BCL2L2 binding generated through the RNA hybrid. Luciferase activity in the 293T cells that are transiently cotransfected with the miR expression vector for the sense strand of miR-204 and (D) the positive and negative control reporter vectors (PTS and MTS, respectively) or (E) 3′-UTR of the BCL2 reporter vectors (TS and MTS). miR-15a that had been demonstrated to target BCL2 is the positive control. The data represent the mean ± the standard deviation of three independent experiments, each of which was performed in triplicate. mfe = minimum free energy; MTS = mutated targeting site; PTS = positive targeting site; TS = targeting site; UTR = untranslated region. *p < 0.05.
BCL2L2 are displayed in Figure 2C. To substantiate that miR-204 binding to the BCL2 and BCL2L2 binding sites was calculated, using the RNA hybrid, as \(-19.4\) kkal/mol and \(-18.3\) kkal/mol, respectively. These results further emphasized that the targeting sequence within the BCL2 3’-UTR is highly likely to be miR-204 binding. The predicted structures of miR-204 binding to BCL2 and BCL2L2 are displayed in Figure 2C. To substantiate that miR-204 directly regulates BCL2, we first tested whether the pSM-derived vectors generated functional matured miRNAs. As shown in Figures 2D and 2E, lucerase activity was reduced significantly in the cells that were cotransfected with pSM-204 and pmiR-204PTS and cells cotransfected with pSM-15 and pmiR-15PTS. None of the miRNA expression plasmids had a regulatory effect on the pmiR-GLO reporter vector with the MTSS (Figure 2D). When replaced, the reporter vectors to pmiR-Bcl2-204TS and pmiR-Bcl2-15TS bore the BCL2 targeting sequence of miR-204 and miR-15a, respectively, the lucerase activities were decreased (Figure 2E).

We cloned reporter vectors (pmiR-Bcl2-204MTS and pmiR-Bcl2-15MTS) containing the reversed seed sequences of miR-204 and miR-15a on the 3’-UTR of BCL2 as the MTs. As anticipated, these mutants eliminated the interaction between miR-204 and miR-15a with the 3’-UTR of BCL2 (Figure 2E). These results indicated that pSM-204 and pSM-15 generated functional and matured miRNAs that directly interacted with the 3’-UTR of BCL2.

3.3. Decreased levels of BCL2 mRNA and protein in pSM-204 transfected T24 cells

We tested the level at which BCL2 was downregulated by the elevated miR-204 because miRNAs may suppress the expression of a specific target by reducing mRNA stability or blocking translation. The expression of BCL2 mRNA in the pSM-204 transfected cells was measured through Q-PCR at 48 hours post-transfection. The BCL2 mRNA expression levels were reduced to 52.01% ± 8.81%, 35.12% ± 6.18%, and 51.03% ± 2.21% in the T24 cells, 293T cells, and HeLa cells, respectively, compared with the vector-only control (i.e., pSM; Figure 3A, left histogram). As a positive control, the mRNA expression of BCL2 was reduced to 64.31% ± 4.81%, 50.13% ± 3.71%, and 55.81% ± 6.21% in the T24 cells, 293T cells, and HeLa cells, respectively (Figure 3A, right histogram).

We next examined the BCL2 protein level in response to the elevated miR-204 expression. As displayed in Figure 3B, the BCL2 level was reduced in the pSM-204 transfected cells, compared with the vector-only control (T24 cells, 54.67% ± 18.23%; 293T cells, 28.35% ± 9.52%). These data suggested that miR-204 suppressed BCL2 expression in RNA and in the protein.

3.4. Suppression of BCL2 by miR-204 induced apoptosis and reduced cell viability in T24 cells

BCL2 is an antiapoptotic protein that inhibits apoptosis by interacting with proapoptotic members of the BCL2 family such as BAD, BAX, and BID. Decreased BCL2 expression disrupts the balance of anti- and proapoptotic homodimers, and thus activates an apoptotic cascade by disrupting the mitochondria. We thus investigated the induced apoptosis in pSM-204-transfected cells by detecting the cytochrome c release, cleavage of caspase 3, and activation of caspase 3/7 activities. As illustrated in Figure 4A, cytochrome c was released from the mitochondria to the cytosol in pSM-204-transfected T24 cells, which indicated that miR-204 targeting of BCL2 causes an imbalance in anti- and proapoptotic protein levels and creates a mitochondria-dependent apoptotic pathway. Furthermore, the expression of BAD and BAX was unaffected, whereas the expression of cleaved-caspase 3 was increased in the pSM-204-transfected cells (Figure 4B). Furthermore, caspase 3/7 activation was also detected in cells that were transfected with pSM-204, which indicated that it could be suppressed by cotransfection with reporter constructs (Figure 4C). These results confirm that BCL2 downregulation by miR-204 triggers mitochondria-dependent apoptosis, and that miR-204 expression is crucial for this mechanism. To investigate the biological importance of BCL2 as a target of miR-204, we measured the cell viability in the pSM-204 transfected cells. As shown in Figure 4D, cell viability was reduced in cells that were transfected with pSM-204 (T24 cells, 62.10% ± 5.18%; 293T cells, 53.12% ± 2.34%), compared with the vector control.

To further confirm that miR-204 reduces cell viability by targeting BCL2, a rescue experiment was performed by cotransfecting the reporter constructs harboring the miR-204-positive targeting sequences (i.e., pmiR-204PTS) or the 3’-UTR of BCL2 (i.e., pmiR-BCL2-204-4TS). These reporters generated miR-204-targeting sequences to compete with exogenous miR-204 or endogenous BCL2 transcripts. As displayed in Figure 4D, cotransferring pSM-204 with pmiR-204PTS restored cell viability in the T24 and 293T cells, whereas cotransfecting pSM-204 with negative controls partially rescued cell viability. These results indicated that miR-204 targets BCL2 and reduces cell viability; however, other mRNA targets of miR-204 may also have contributed to the loss in viability because cell viability was not fully recovered in the cells that were cotransfected with pmiR-Bcl2-204TS and pSM-204.

4. Discussion

The imbalance of anti- and proapoptotic protein is a critical mechanism of cancer progression. Upregulation of BCL2 has been demonstrated in numerous cancer types, and high BCL2 expression has been detected in androgen-independent prostate cancer. Therefore, BCL2 is a potentially viable therapeutic target in cancer treatment. Downregulation of miR-204 has been reported in human breast cancer, renal clear cell carcinoma, head and neck tumors, leukemia, and endometrioid adenocarcinoma. In the present study, we showed that forced expression of miR-204 in cancer cells such as T24 and 293T cells, induced apoptosis by directly targeting BCL2. These results indicated that the interaction between miR-204 and BCL2 may be universal. For the positive control, we also demonstrate that another antiapoptotic protein, BCL2L2, was targeted by miR-204.

The elevation of miR-204 expression significantly reduced the cell viability of T24 and 293T cells. Furthermore, the viability of these cells was fully restored and partially restored by cotransfcting reporter constructs bearing miR-204 PTS (pmiR-204PTS) and a BCL2 targeting site (pmiR-Bcl2-204TS), respectively. These results indicated that BCL2 is not the only target of miR-204 that reduces cell viability. BCL2L2 is reportedly an antiapoptotic target of miR-204 in human trabecular meshwork (HTM) cells. The elevation of miR-204 in HTM cell lines also induced apoptosis and caused cell death. This further supported the notion that miR-204 functions as a tumor-suppressing miRNA by targeting antiapoptotic genes.

In conclusion, our results revealed that BCL2 is a direct target of miR-204. Consistent with the decrease in BCL2 expression, miR-204 overexpression resulted in significant alteration in cancer cells such as increased apoptosis and cell death. Our results specifically confirmed the direct targeting of miR-204 to the 3’-UTR of BCL2 and demonstrated a significant decrease in BCL2 expression by miR-204. These data collectively suggested that decreased miR-204 expression in cancers may increase BCL2 expression and promote cancer progression. The induction of apoptosis by miR-204 in these
cancer cells provided further evidence that miR-204 suppresses cancer growth, even in miR-204-positive cancer cells (i.e., 293T cells). Hence, future clinical studies that aim to elucidate the relationship between miR-204 and antiapoptotic genes may provide further insight into the design of novel molecular therapies for BC, and, more importantly, in the inhibition of cancer progression and subsequent low overall survival. The findings of this study offer insight into an innovative approach that utilizes the miRNA...
pathway (i.e., the bladder maximizes the effect of gene therapy by using miRNAs because of its accessibility and closed environment).

**Conflicts of interest**

The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

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