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ORIGINAL ARTICLE

MicroRNA-15 regulates the proliferation, migration and invasion of thyroid cancer cells by targeting Bcl-2

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Summary

Purpose: *Thyroid cancer causes significant mortality and 1-1.5% of all the new diagnosed cancers are thyroid cancers. The incidence of thyroid cancer is increasing at an alarming rate and therapeutic targets are lacking. This study was undertaken to investigate the role and therapeutic implications of microRNA (miR)-15 in thyroid cancer.*

Methods: *Expression analysis was performed by qRT-PCR. Transfections were performed by Lipofectamine 2000 reagent. The cell viability was determined by MTT assay. Apoptosis was detected by acridine orange (AO)/ethidium bromide (EB). The percentage of apoptotic cells was estimated by annexin V/ propidium iodide (PI) staining. Wound healing and transwell assays were used to monitor the cell migration and invasion. Protein expression was determined by western blotting.*

Results: *The expression of miR-15 was found significantly decreased in thyroid cancer cells. Ectopic expression of miR-15 promoted the apoptosis of MDA-T35 thyroid cancer cells. The percentage of apoptotic MDA-T35 cells was 1.9% in thyroid cancer and 40.1% in miR-15 mimics transfected cells. The apoptosis promoted by miR-15 overexpression was also associated with enhancement of Bax and depletion of Bcl-2 in MDA-T35 cells. The TargetScan analysis showed Bcl-2 to be the target of miR-15. Additionally, the expression of Bcl-2 was also enhanced in all the thyroid cancer cells and miR-15 ectopic*
expression could cause suppression of the Bcl-2 expression in MDA-T35 cells. The wound healing assay showed that miR-5 overexpression caused decrease in the migration of MDA-T35 cells while the transwell assays showed decline in the invasion of miR-15 mimics transfected MDA-T35 cells.

Conclusion: *To sum up, miR-15 may exhibit therapeutic implications in thyroid cancer and may prove useful in thyroid cancer treatment.*

Key words: *thyroid cancer, microRNA, apoptosis, Bcl-2, migration*

Introduction

Thyroid cancer accounts for 1-1.5% of all the newly diagnosed cancers in United States [1]. Although the incidence of most head and neck cancers has decreased over the last few decades, the incidence of thyroid cancer is still steeply increasing. Genetic and environmental factors have been held responsible for the increasing incidence of thyroid cancer [2]. Moreover, recent studies have indicated thyroid cancer to be more common in women than in men [3]. Surgical interventions, chemotherapy and radiotherapy are employed for the treatment of thyroid cancer [4]. Nonetheless, late diagnosis due to the lack of biomarkers and lack of therapeutic targets/agents create problems to the treatment of thyroid cancer [5]. A number of studies carried out have shown that microRNAs (miRs) may exhibit therapeutic implications in treating human diseases such as cancer [6]. MiRs are nucleotides

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around 19-23 in length and regulate the expression of target genes via post-transcriptional regulation [7]. The miR-15 has been shown to control diverse arrays of molecular processes [8]. In chronic lymphocytic leukemia cells, miR-15 has been reported to suppress proliferation via induction of apoptotic cell death [9]. In prostate cancer, miR-15 regulates the growth by modulating the expression of multiple targets [10]. Similarly, miR-15 has been shown to regulate cisplatin-resistance in cancer cells [11]. In yet another study, miR-15 has been shown to control multidrug resistance in gastric cancer cells [12]. However, there is no report on the role and therapeutic implicates of miR-15 in thyroid cancer. Therefore, this study was undertaken to ascertain the role and therapeutic implications of miR-15 in thyroid cancer. The main purpose of the current study was to investigate the role of miR-15 in cell proliferation, migration and invasion as well as in Bcl-2 expression in thyroid cancer.

Methods

Cell culture conditions

The thyroid cancer cell lines (MDA-T32, TT, K1 and MDA-T68) and the normal thyrocytes were obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Massachusetts, USA),100 µg/ml streptomycin and 100 U/ml penicillin G (Himedia, Pennsylvania, USA) in an incubator at 37°Cwith 5% CO_2.
Expression analysis

The TRIzol reagent (Invitrogen) was used for extracting RNA from the tissues and cell lines. This was followed by purification of the RNA by RNeasy Mini Kit (Qiagen). The complementary DNA (cDNA) was then synthesized with the help of miScript Reverse Transcription Kit (Qiagen). Afterwards, the cDNA was amplified using SYBR Premix Ex *Taq*™ (TaKaRa, Otsu, Shiga, Japan). The expression was estimated by $2^{-\Delta\Delta Ct}$ method and actin was used as internal control. Heat map was generated by the online heatmaper software (http://www2.heatmapper.ca/).

Cell transfection

The miR-15 mimics and normal control (NC) were synthesized by RiboBio (Guangzhou, China). The transfection was then carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instruc¬tions. As the MDA-T35 cells reached 80% confluence, the appropriate concentrations of miR-15 mimics or NC was transfected into these cells.
Cell viability

For assessment of cell viability, the MDA-T35 cells were transfected and incubated for 24 h and then incubated with MTT (500 µg/mL) for 4 h. Dimethylsulphoxide (DMSO) (10%) was then added to dissolve the blue formazan crystals formed. Finally, the optical density (OD) was taken at 570 nm and cell viability estimated as the percentage of the control.

AO/EB staining assay

The MDA-T32 cells (0.6×10^6) were cultured in 6-well plates. Following incubation of around 24 h at 37°C, 25 µl of cell culture were put onto a glass slide and stained with a 1 µL solution of AO/EB. The slides were covered with cover slips and examined under fluorescence microscope.

Annexin V/PI staining assay

ApoScan kit was used to determine the apoptotic MDA-T32 cell percentage. In brief, transfected MDA-T32 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC or PI. The percentage of apoptotic MDA-T32 cells at each concentration was then determined by flow cytometry.

Target identification and dual-luciferase reporter assay

The miR-15 target was identified by TargetScan online software (http://www.targetscan.org). The miR-15 mimics or NC were co-transfected with Plasmid pGL3-BCl-2´-UTR-WT or pGL3-BCl-2´-UTR-MUT into MDA-T32 cells. Dual-luciferase reporter assay (Promega) was carried out at 48 h after transfection. *Renilla* luciferase was used for normalization.

Transwell assay

The effects of miR-15 overexpression on the invasion ability of MDA-T35 cells was determined by transwell chambers (8 mm pore size, Corning, NY, USA) with Matrigel (Millipore, Billerica, USA). The MDA-T32 cells were transfected with miR-15 mimics and NC and around 200 ml of cell culture were placed onto the upper chambers and only the medium was placed in the bottom wells. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200× magnification.

Wound-healing assay

After 24 h of miR-15 mimics and NC of transfection into MDA-T35 cells, Dulbecco's Modified Eagle Medium (DMEM) was removed and the cells were subjected to PBS washing. A sterile pipette tip was employed to scratch a wound in each well, the cells were washed again and a picture was taken. The plates were cultured for 24 h and a picture was taken again under an inverted microscope (Leica, Germany).

Western blotting

The transfected MDA-T35 cells were harvested with centrifugation. Then, the cells were lysed in lysis buffer containing protease inhibitor. About 45 µg of protein

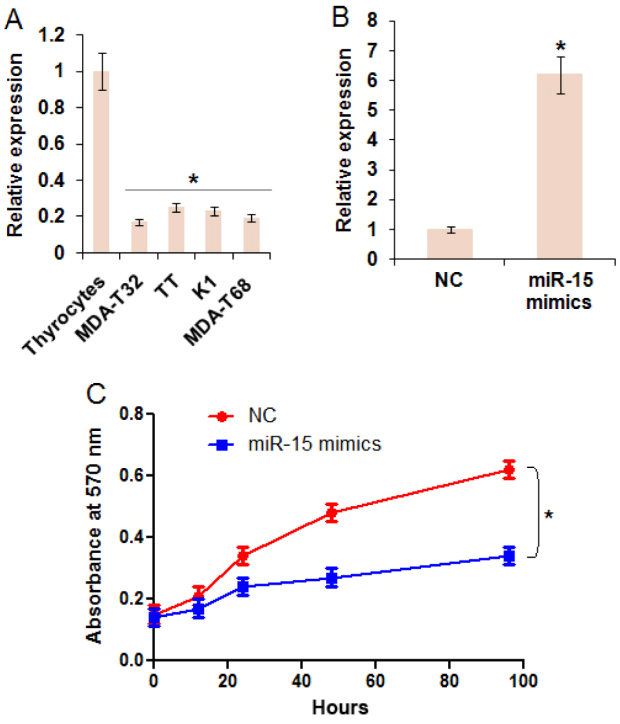

Figure 1. miR-15 suppresses the proliferation of thyroid cancer cells. **(A):** Expression of miR-15 in different thyroid cancer cells and normal thyrocytes. **(B):** Expression of miR-15 in NC or miR-15 mimics transfected MDA-T32 cells as determined by qRT-PCR. **(C):** MTT assay showing the viability of NC or miR-15 mimics transfected MDA-T32 cells. The experiments were performed in triplicate and presented as mean ± SD (*p<0.05).

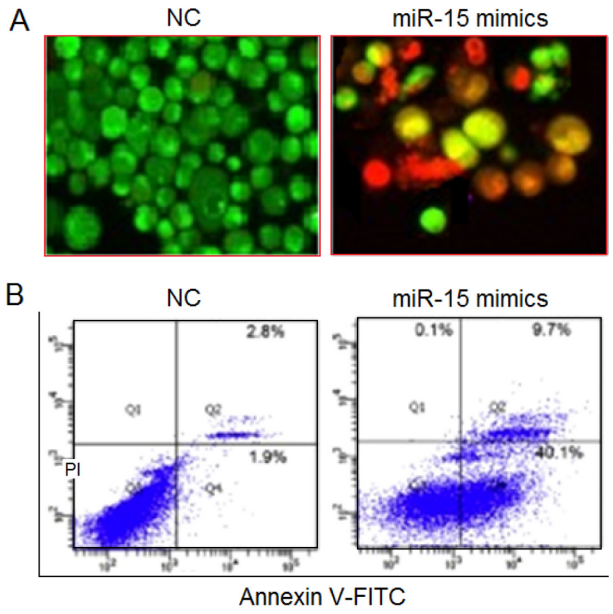

Figure 2. miR-15 triggers apoptosis in the MDA-T32 thyroid cancer cells. **(A):** AO/EB assay of NC or miR-15 mimics transfected MDA-T32 cells. **(B):** AO/EB assay of NC or miR-15 mimics transfected MDA-T32 cells showing induction of apoptosis. The experiments were performed in triplicate. Green color indicates normal living cells while yellow and red colors indicate apoptotic cells.

was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride (PVDF) membrane. The membranes were then treated with tris-buffered saline (TBS) an exposed to primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

SPSS 16.0 software (IBM, Chicago, Ill, USA) was used for statistical analyses and the results were expressed as mean±SD of three independent experiments.

Student's *t*-test was used for comparison between 2 samples and one way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were used for comparisons between >2 samples. P<0.05 indicated a statistically significant difference.

Results

miR-15 is downregulated in thyroid cancer cells

The expression of miR-15 was investigated in different thyroid cancer cells by qRT-PCR and the results showed that miR-15 was significantly decreased in the thyroid cancer cells as compared to the normal thyrocytes (Figure 1A). The expression of miR-15 was 9-fold lower in the thyroid cancer cells. The MDA-T35 showed the lowest expression (9-fold downregulation) of miR-15 among all the cell lines.

miR-15 suppressed the proliferation of MDA-T35 thyroid cancer cells

To ascertain the effects of miR-15 overexpression on the proliferation of the MDA-T35 thyroid cancer cells, the miR-15 mimics transfected MDA-T35 cells were subjected to MTT assay. The overexpression of miR-15 was confirmed by qRT-PCR

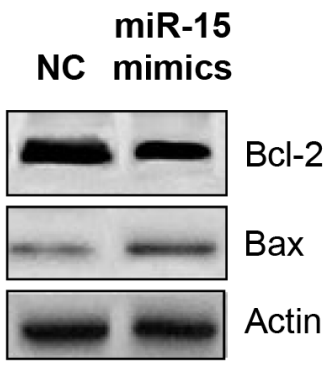

Figure 3. Expression of Bax and Bcl-2 in NC and miR-NC transfected MDA-T32 cells as determined by western blot analysis. The experiments were performed in triplicate. Bax expression increased while Bcl-2 expression decreased after treatment.

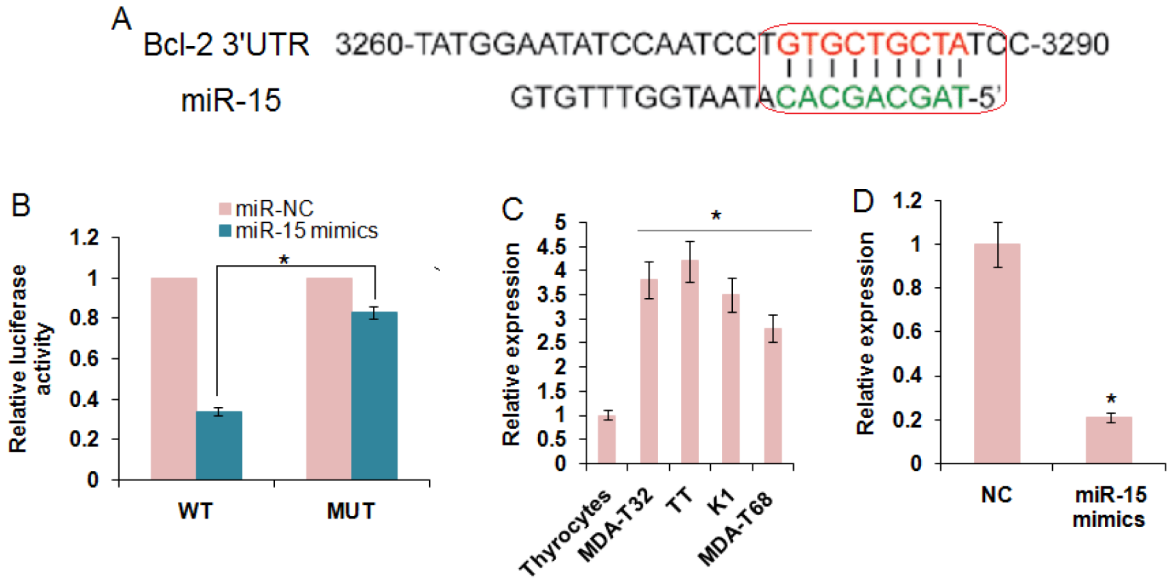

Figure 4. miR-15 targets Bcl-2 in thyroid MDA-T32 cells. **(A):** TargetScan analysis showing the Bcl-2 as the target of miR-15. **(B):** Dual luciferase assay confirming Bcl-2 as target of miR-15. **(C):** Relative expression of Bcl-2 in thyrocytes and thyroid cancer cells. **(D):** Relative expression of Bcl-2 in NC or miR-15 mimics transfected MDA-T32 thyroid cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

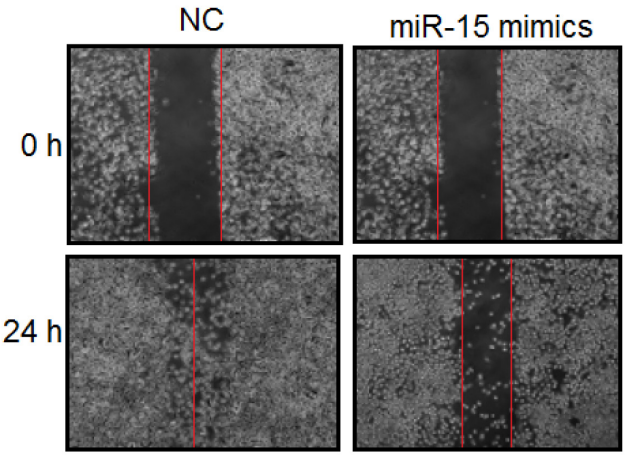

Figure 5. Wound healing assay showing cell migration in NC or miR-15 mimics transfected MDA-T35 cells. The experiments were performed in triplicate. miR-15 caused significant decline in the migration of MDA-T35 cells as evidenced for the scratch width.

which showed 6.2-fold increase in miR-15 expression (Figure 1B). Thereafter, the cell viability was assessed at different time intervals. It was found that miR-15 overexpression caused significant decline in the viability of the MDA-T35 cells (Figure 1C).

miR-15 promoted apoptosis in the MDA-T35 cells

To ascertain the reasons underlying the inhibition of proliferation triggered by miR-15 overexpression, the miR-15 mimics transfected MDA-T35 cells were subjected to AO/EB and Annexin V/PI staining. The results of AO/EB staining revealed that transfection of the MDA-T35 cells with miR-15 mimics triggered apoptotic cell death (Figure 2A). Annexin V/PI staining showed that the percentage of the apoptosis in miR-15 mimics transfected MDA-T35 cells was 40.1% as compared to the 1.9% in NC transfected cells (Figure 2B). The ectopic expression of miR-15 also resulted in enhancement of Bax expression and depletion of Bcl-2 expression (Figure 3).

miR-15 targeted Bcl-2 in MDA-T35 thyroid cancer cells

The TargetScan analysis showed that miR-15 targets Bcl-2 in MDA-T35 thyroid cancer cells (Figure 4A). Dual luciferase also confirmed Bcl-2 as the target of miR-15 (Figure 4B). Therefore, the expression of Bcl-2 was also determined in all the thyroid cancer cells. The results showed that the expression of Bcl-2 was significantly increased in all the thyroid cancer cells (Figure 4C). The fold upregulation of Bcl-2 ranged between 2.8 to 4.2 in thyroid cancer cells. Nonetheless, miR-15 overexpression caused suppression of Bcl-2 in MDA-T35 cells (Figure 4D).

miR-15 inhibited the migration and invasion of MDA-T35 cells

The wound healing assay was used to investigate the effects of miR-15 on the migration of MDA-T35 cells. The results showed that miR-35 caused significant decline in the migration of the MDA-T35 cells as evidenced from the scratch width

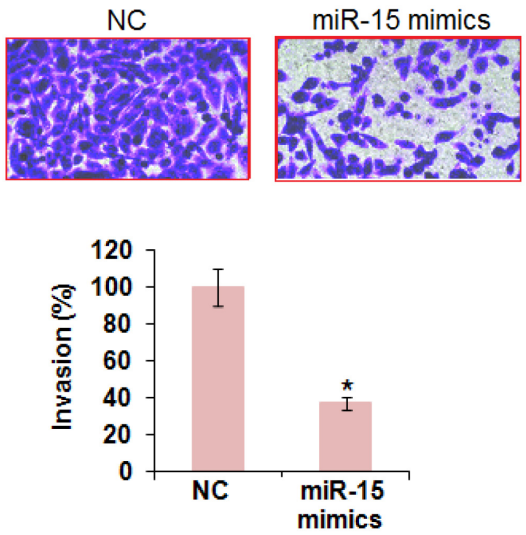

Figure 6. Transwell assay showing the cell invasion in NC and miR-15 mimics transfected MDA-T32 cells. The experiments were performed in triplicate and expressed as mean ± SD. miR-15 significantly suppressed the invasion of MDA-T35 cells (*p<0.05).

(Figure 5). The transwell assay also showed that miR-15 significantly suppressed the invasion of the MDA-T35 cells. The migration of the MDA-T35 cells was suppressed by up to 63% upon miR-15 overexpression (Figure 6).

Discussion

Thyroid cancer causes considerable mortality around the world. The lack of the reliable and efficient therapeutic targets/agents is one of the major obstacles in the treatment of thyroid cancer [13]. The diverse role that miRs play in humans by controlling the expression of approximately 30% of the human genes indicates that miRs may prove useful therapeutic targets for treating human diseases including cancer. Herein, we investigated the role of miR-15 in thyroid cancer [14]. The results indicated that the expression of miR-15 is significantly increased in thyroid cancer cells. In pancreatic cancer, miR-15 has also been shown to alter the findings of the present study [15]. These findings are all in agreement with previous observations wherein miR-15 has been shown to regulate the growth of a diversity of cancer cells. In ovarian cancer cells, miR-15 targets Bim-1 to modulate their proliferation [16]. In non-small lung cancer cells, miR-15 controls the cell proliferation by regulating the cell cycle check points [17]. Similarly, in breast cancer cells, miR-15 modulates the expression of p70S6 kinase1 to regulate their growth [18]. A previously carried out research had shown that miR-15 triggers apoptosis in chronic lymphocytic leukemia cells [9]. Consistently, we performed AO/EB staining and also annexin V/PI staining of the miR-15 mimics-transfected thyroid cancer cells to ascertain if miR-15 overexpression induces apoptosis. Intriguingly, it was found that ectopic expression of miR-15 promotes apoptotic cell death of the MDA-T35 thyroid cancer cells. Moreover, miR-15 mimics promoted apoptosis in the MDA-T35 thyroid cancer cells and was also associated with overexpression of Bax and suppression of Bcl-2 expression, further confirming our findings. MiRs have been shown to perform their functions by modulating the expression of their target genes [9,19-21]. Herein, we found that miR-15 targets Bcl-2 in thyroid cancer cells and overexpression of miR-15 suppresses the Bcl-2 expression. To ascertain the impact of miR-15 on the migration and invasion of the MDA-T35 thyroid cancer cells, wound healing assay and transwell assay were performed which showed that miR-15 overexpression suppresses the migration and invasion of the MDA-T35 cells. This observation is also in agreement with a previous research wherein miR-15 regulates the migration and epithelial-mesenchymal transition of the tongue cancer cells [21].

Conclusion

The findings of the present study indicate that miR-15 regulates the proliferation of the thyroid cancer cells by suppressing the expression of Bcl-2. Furthermore, miR-15 also regulates the migration and invasion of the thyroid cancer cells. Therefore, miR-15 may exhibit therapeutic implications in thyroid cancer and may prove useful in thyroid cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

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