Original Article

MicroRNA-7 regulates the proliferation and metastasis of human papillary carcinoma cells by targeting Bcl-2

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Abstract: Papillary carcinoma is the most common type of thyroid cancer responsible for significant number of mortalities across the globe. This study was conducted to investigate the role and therapeutic implications of microRNA-7 in human papillary carcinoma. Gene expression analysis was carried out through quantitative real time PCR method. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to determine the cell proliferation. Clonogenic assay was used to assess the colony forming ability of cancer cells. Cell apoptosis was analyzed by 4',6-diamidino-2-phenylindole (DAPI), acridine orange/ethidium bromide (AO/EB) and annexin V/PI staining assays. Migration of cancer cells was estimated through scratch heal assay and cell invasion was determined by transwell assay method. Western blotting was done to examine the protein expression. Xenografted mice models were employed to examine the effects of miR-7 overexpression in vivo. Results showed miR-7 to be significantly (P < 0.05) repressed in papillary carcinoma. Cancer cell proliferation was inhibited by miR-7 through induction of apoptotic cell death as revealed by DAPI, AO/EB and annexin V/PI staining assays. The colony forming potential of cancer cells also decreased under miR-7 overexpression. miR-7 overexpression also inhibited the migration and invasion of cancer cells. Bcl-2 was identified as the intracellular target of miR-7 and regulatory effects of miR-7 were seen to be exerted through translation repression of Bcl-2. The results of xenograft study revealed miR-7 overexpression significantly (P < 0.05) suppressed the growth of the tumor in vivo. The results point towards the therapeutic implications of miR-7 in the management of papillary carcinoma.

Keywords: MicroRNA, papillary carcinoma, flow cytometry, apoptosis, migration, invasion, xenografted mice

Introduction

Among the human endocrine system related malignancies, the thyroid cancer is the most prevalent disorder [1]. The papillary carcinoma is the most common type of thyroid cancer [1]. Researchers have revealed that a genetic mutation in BRAF gene is responsible for onset and progression of papillary carcinoma in most of the cases [2]. However, there are also reports have revealed the involvement of lifestyle and environmental factors in the development of papillary carcinoma [3]. The molecular mechanisms of papillary carcinoma is somewhat poorly understood. The investigations of recent times have shown that some non-coding RNA families play profound role in human diseases including cancer development and proliferation [4]. Among these non-coding RNAs, microRNAs (miRs) have received tremendous attention from scientific community. The miRs are about 22 nucleotide long RNA molecules which are synthesized in higher organisms and perform their regulatory role at translational level by acting as repressors [5-7]. The miRs target and repress the mRNA transcripts of specific genes [8, 9]. The miRs have been found to be involved in number of human malignancies and their involvement in human cancers is well recognized [10-12]. Human cancers proceed with alteration in miR levels and thus the changes in levels of specific miRs have been proposed to be employed for diagnosis of particular type of cancer onset [13]. In an effort, we investigated the regulatory role of microRNA-7 (miR-7) in human papillary carcinoma. The results showed...
that miR-7 levels are downregulated in human papillary carcinoma. Further, the overexpression of miR-7 in cancer cells reduced the cell proliferation considerably by inducing cellular apoptosis. The cancer cell migration and invasion was also inhibited remarkably. The intracellular target of miR-7 was shown to be apoptosis related protein Bcl-2. The Bcl-2 protein levels fall under miR-7 overexpression and miR-7 was seen to exert its regulatory role by interacting and repressing mRNA levels of Bcl-2. Moreover the in vivo study in xenografted mice revealed that miR-7 negatively regulated the growth of tumor development, further supporting its anti-cancer role against the human papillary carcinoma. Collectively, the study showed miR-7 as an important regulatory molecule and points towards its therapeutic implications for the treatment of human papillary carcinoma.

Materials and methods

Culture and transfection of cancer cells

Normal human thyroid epithelial cells, Nthy-ori 3-1 and papillary cancer cell lines, TPC-1, IHH-4 and BCPAP were purchased from Shanghai Institute of Cell Biology, China. Culturing of cell lines was done in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS. Cultures were maintained in humidified incubator at 37°C with 5% CO\textsubscript{2}. Cancer cells were transfected using Lipofectamine 2000 reagent (Invitrogen). Following transfection the cells were trypsinized at 80% confluence to obtain a homogenous cell suspension. miR-7 NC, miR-7 mimics, si-NC and si-Bcl-2 constructs were ordered from RiboBio company. pcDNA3.1 mammalian expression vector was used for transformation of Bcl-2 gene in cancer cells. The study was approved by the research ethics committee of the institute under the approval number PMC-210IV-2019.

RNA isolation and expression analysis

Following standard protocol, total RNA was isolated using TrizOL reagent (Invitrogen). Post RNA isolation, DNAse I (Invitrogen) treatment was given to remove DNA contamination. The RNA was reverse transcribed to synthesize cDNA using RevertAid First Strand Synthesis kit (Invitrogen). cDNA synthesis was confirmed by β-actin PCR. The expression analysis was performed by quantitative RT-PCR method on QuantStudio 3.0 Real Time PCR system (Applied Biosystems) through SYBR Green method. Human GAPDH gene was used as an internal control in gene expression studies. The nucleotide sequences of real time primers were miR-7: Forward 5'-TGGAAAGACTAGTATTTGTTGT-3' and Reverse from miScript SYBR Green PCR Kit, Bcl-2: Forward 5'-GTGTGTGGAGAGCGTCAAC-3' and Reverse 5'-GAGCAGAGCTCTCAGAAGACGC-3'; GADPH: Forward 5'-AGTCGGTGTGAAACGATTGG-3' and Reverse 5'-GGGTGCTGGTATGCAACA-3'.

Cell proliferation assay

Using hemocytometer, cell counting of transfected cells was carried out and about 2 × 10\textsuperscript{5} cells were added to each well of 96 well plate followed by an incubation of 37°C for 24 h. Then 100 μl of 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent was put into each well and incubation of 4 h at 37°C was given. Following, 150 μl DMSO (Sigma-Aldrich) was added to each well to solubilize the product, i.e., formazan. Each sample was processed for OD measurement at 570 nm to assess the cell proliferation.

Clonogenic assay

To analyze the colony forming ability of transfected TPC-1 cancer cells, 200 cells were added to each well of 6-well plate and cultured for 6 days. Then, the cells were harvested and washed with PBS. The cells were fixed with 70% ethanol and stained with crystal violet solution. Finally, the colony assessment was done and pictures were taken. Percent number of colonies was estimated for at least 10 random fields.

Analysis of apoptosis

TPC-1 cancer cells were transfected for with miR-7 NC and miR-7 mimics and incubated at 37°C for 24 h. The cells were then added to 6-well plate and cultured till density of 1 × 10\textsuperscript{6} cells/well was reached. Afterwards, the cells were harvested by centrifugation and washed with PBS. The cells were then fixed using 70% ethyl alcohol and subsequently stained with 4',6-diamidino-2-phenylindole (DAPI) or a solution of acridine orange/ethidium bromide (AO/EB). Following, the cells were processed for
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fluorescent microscopy to examine the nuclear fluorescence. The cells were also processed for flow cytometry to better estimate the level of apoptotic cell death after dual staining with Annexin V-FITC/PI mix.

**Scratch assay**

The papillary cancer cells transfected for 24 h were cultured in 6-well plate to obtain a cellular density of $2 \times 10^5$ cells/well. The cells were starved to obtain a uniform cell monolayer. Using 100 µl pipette tip, a scratch line was drawn on this monolayer whose width was noted down. This was followed by an incubation of 37°C for 24 h. The width of the scratch was again observed to analyze the cell migration.

**Transwell assay**

Transwell assay was performed for determining the invasion of TPC-1 cancer cells transfected with miR-7 NC and miR-7 mimics. In a few words, the TPC-1 cells were put into the upper chamber of transwell plate coated with matrigel and only RPMI-1640 culture medium was kept in the lower well. The plate was incubated at 37°C for 24 h. Cancer cells invading lower chamber were fixed with absolute methanol and stained using crystal violet. Inverted high magnification microscope was used to visualize the invaded cells and photographs were taken. At least ten random fields were used for analyzing the percent cell invasion.

**Target prediction of miR-7 and interaction analysis**

TargetScan (http://www.targetscan.org) online software was used to predict the regulatory target of miR-7. The prediction was confirmed by further in silico analysis. Also, the wild type (WT) and mutant (MUT) stretches designed and ordered for RiboBio were co-transfected with miR-NC or miR-7 mimics for interactional study using Dual-Luciferase® Reporter (DLR™) Assay System (Promega). The assay was carried out as per manufacturer protocol using Renilla-luciferase activity to normalize the results.

**Western blotting**

RIPA lysis buffer was used to obtain the cell lysates of cancer cells which were homogenized using trypsin. The total protein estimations were made using Bradford assay. Exactly, 45 µg protein was loaded from each lysate sample on SDS-PAGE. The gel was then blotted to nitrocellulose membrane which was processed for primary and secondary antibody exposure. Finally, enhanced chemiluminescent reagent was used to analyze the proteins of interest. Human GADPH protein was used as an internal control in blotting study.

**In vivo xenograft study**

The nude male xenografted BALB/c mice (5 weeks old) were obtained form Peking Union Medical College. The animal care and usage for experimentation purpose were done following the strict institutional ethical guidelines. The TPC-1 cancer cells were transfected with miR-7 mimics or miR-NC for 24 h. The transfected cells were cultured until the cellular growth of $1 \times 10^7$/ml was achieved. At this stage, the cells were harvested and dissolved in a mixture of ethanol:saline:PEG5000 (2:3:4) used a vehicle following which 100 µl of the vehicular mix with $5 \times 10^6$ cells was injected into the right inguinal area of the mice, subcutaneously. After one week, the mice were divided randomly into two groups with ten mice in each group. The group one constituted the mice injected with miR-NC transfected cancer cells (control) and second group was of those mice which were injected with miR-7 mimics transfected cancer cell mix. Mice were continuously monitored for a period of 6 weeks (with day 1 the day on which the mice were injected). On the day after six weeks, the mice were sacrificed and tumors were excised from five mice belonging to each group, photographed and subsequently weighed. The size of tumors was determined using Vernier calipers.

**Immuno-histochemical staining**

The expression of specific proteins in tissue samples was further analyzed by immuno-histochemical (IHC) staining procedure. Here, the samples formalin fixed, paraffin embedded, sectioned to obtain sections with 4 µm thickness and then stained with hematoxylin and eosin. The mouse tumor tissues were immune-stained after removal of paraffin using monoclonal antibodies against Ki67 and cleaved caspase-3. The sections were hydrated with alcohols and the quenching of endogenous peroxidase was performed in methanol. They
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were then transferred to the Dako Autostainer and incubated for 30 min with detection system and finally visualized by fluorescent microscope for staining analysis.

Statistical data validation

At least three replicates were used for each experimental procedure to minimize the experimental error. Final values were presented as mean ± SD. Further, t-test was performed with the help of GraphPad prism 7 software to examine the statistical difference between two values where a *P < 0.05 was taken as measure of statistically significant difference.

Results

miR-7 is suppressed in papillary carcinoma

Quantitative real time expression analysis of miR-7 in cancer cell lines TPC-1, IHH-4, BCPAP and normal thyroid epithelial cells, Nthy-ori 3-1 revealed that the transcript abundance of miR-7 is significantly lower in cancer cells (Figure 1A). This suggests that the human papillary carcinoma proceeds with suppression of miR-7 transcripts. Interestingly, the mRNA level was lowest in TPC-1 cells and thus they were used for further characterization.

miR-7 inhibited proliferation of cancer cells through apoptosis

To understand the regulatory effect of miR-7 on the proliferation of cancer cells, overexpression of miR-7 was performed by transfection method. The overexpression of miR-7 was confirmed by RT-PCR (Figure 1B). TPC-1 cancer cells were transfected for 0, 24, 48, 72, 96 and 120 h with miR-NC and miR-7 mimics. The proliferation rates were estimated using OD_{570} nm absorbance values. The results indicate that miR-7 overexpression inhibited the proliferation of TPC-1 cells and at higher transfection levels, the inhibition was comparatively more prominent (Figure 1C). The colony forming assay further supported the observation. The colony number was comparatively very small for cancer cells transfected with miR-7 mimics (Figure 2A). The percentage of cancer cell colonies was only about 37% under miR-7 overexpression in comparison to negative control set up (miR-NC) (Figure 2B).

To draw an inference whether the decline in cell proliferation was due to apoptosis of cancer cells, nuclear morphology of TPC-1 cancer cells were examined through DAPI staining. It was observed that viable cell count was significantly lower under miR-7 overexpression (Figure 2C). The AO/EB staining assay also pointed towards the induction of apoptosis (2D). Annexin V/PI staining showed that apoptotic TPC-1 cells increased significantly upon miR-7 overexpression (Figure 2E). The flow cy-
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The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

Figure 2. miR-7 induces apoptosis in TPC-1 cancer cells. A. Colony formation by TPC-1 cells transfected with miR-NC and miR-7 mimics. B. Percentage of colonies formed by TPC-1 cancer cells transfected with miR-NC and miR-7 mimics. C. DAPI staining of cancer cells transfected with miR-NC and miR-7 mimics. D. AO/EB staining of cancer cells transfected with miR-NC and miR-7 mimics. E. Flow cytometry for cell apoptosis assessment of TPC-1 cancer cells transfected with miR-NC and miR-7 mimics. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

tometry results were also in coherence with DAPI staining where relative abundance of apoptotic cells was significantly higher in case of miR-7 mimics transfection. Hence, it can be stated that miR-7 reduces the proliferation of papillary cancer cells by inducing apoptotic cell death.

Cancer cell motility is restricted by miR-7

The migration and invasion of TPC-1 cancer cells transfected with miR-NC and miR-7 mimics were determined by scratch heal and transwell methods, respectively. The migration of cancer cells was inhibited under miR-7 mimics transfection, i.e., miR-7 overexpression, considerably (Figure 3A and 3B). Similar was the cancer cell invasion and number of invading cells was significantly lower when miR-7 was overexpressed in cancer cells, being only 39 in terms of percentage values (Figure 3C and 3D). Together, the findings reveal that miR-7 possesses a regulatory potential to restrict the motility of papillary cancer cells, i.e., the cancer cell metastasis.

Bcl-2 is targeted by miR-7 to exert its regulatory effects

The prediction of Bcl-2 as miR-7 target by in silico analysis was based on the complementarity between 938-944 upstream nucleotide bases of 3'-UTR of Bcl-2 mRNA and miR-7 (Figure 4A). The interaction was confirmed by dual luciferase assay where luciferase activity was found to be sufficiently higher for miR-7 and MUT 3'-UTR of Bcl-2 (Figure 4B). Further support was gained from protein expression study of Bcl-2. It was seen that the protein levels of Bcl-2 fall considerably under miR-7 overexpression (Figure 4C). The mRNA levels of Bcl-2 were estimated by RT-PCR in cancer cells and compared with those in normal epithelial
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The mRNA transcripts were significantly higher in cancer cells, further indicating that Bcl-2 is targeted by miR-7 at mRNA level, intracellularly (Figure 4D). Whether the regulatory effects of miR-7 are achieved through Bcl-2 repression, Bcl-2 was silenced in cancer cells through RNA interference. The assessment of proliferation of cancer cells showed that Bcl-2 silencing declined the proliferation rates in the same manner as by overexpression of miR-7 (Figure 4E). Lastly, the inhibitory effects of miR-7 were contradicted when Bcl-2 was overexpressed in cancer cells (Figure 4F). Collectively, the results show that miR-7 targets Bcl-2 inside the cells to repress the mRNA levels leading to decline in Bcl-2 protein, the effects of which are visible as decline in cell proliferation rate through apoptotic cell death.

In vivo tumor growth was inhibited by miR-7

The assessment of papillary tumor growth in xenografted mice showed that the miR-7 overexpression negatively affected the tumor development (Figure 5A). The tumor weight was also inhibited considerably by miR-7 up-regulation (Figure 5B). Same was true for the tumor size. The mice injected with miR-7 mimics transfected cells exhibited significant decline in tumor size (Figure 5C). Additionally, IHC analysis that the expression of Ki67 decreased while as that of cleaved caspase-3 increased upon miR-7 overexpression leading to the inhibition of the tumor growth in vivo (Figure 5C and 5D). These results are supportive of anti-cancer regulatory role of miR-7 against the growth of human papillary carcinoma.

Discussion

Owing to limited success of the currently employed strategies for the treatment of cancer, frequent relapses and adverse effects, considerable research efforts are directed to identify novel therapeutic targets and chemo-
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Figure 4. miR-7 targets and represses Bcl-2 to exert its regulatory effects. A. TargetScan analysis showing Bcl-2 as target of miR-7. B. Dual luciferase reporter assay of miR-7 with WT and MUT 3′-UTR of Bcl-2 mRNA. C. Western blotting for expression of Bcl-2 in TPC-1 cancer cells transfected with miR-NC and miR-7 mimics. D. qRT-PCR analysis of Bcl-2 in normal Nthy-ori 3-1 epithelial cells and three different papillary cancer cell lines. E. MTT assay for assessment of proliferation of TPC-1 cancer cells transfected with si-NC and si-Bcl-2. F. Proliferation of cancer cells transfected with miR-NC, miR-7 mimics and miR-7 mimics+ pcDNA-Bcl-2 by MTT assay. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

therapeutic agents for the management of human cancers [14]. In this regard, different molecular entities are being assessed for their regulatory potential against particular human cancers among which the miRs have also received a tremendous scientific attention in recent years. Researchers have reported that impairment in miR based regulatory mechanics is responsible for development and advancement of number of human malignancies including cancer [14]. The micro RNAs are thus being evaluated for their potential to act as cancer related signature molecules to aid in early prognosis and management of human cancers [15-18]. Here in this study, we showed that the human papillary carcinoma proceeds with repression of miR-7 which is also true for other human cancers like lung and colorectal cancer [19, 20]. Studies have shown that miR-7 is active in reducing the proliferation of cancer cells [21]. Our results also suggest the same. The decline in cell proliferation was attributed to onset of apoptotic cell death in papillary cancer cells and this observation was in conformity with a previous report [22]. Metastasis is one of the most important aspects of cancer growth and helps in progression of cancer through migration and invasion of cancer cells to surrounding tissues from cancer infected area [23]. Interestingly, the results of this study indicate that miR-7 is having a regulatory potential to inhibit the migration and invasion of cancer cells. Such results were also obtained for miR-7 in breast cancer [24]. The miRs perform their
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regulatory functions by repressing the specific targets at post-transcriptional level [25]. Herein, the apoptosis related protein Bcl-2 was shown to be targeted by miR-7. The repression of Bcl-2 at translational level was proved to responsible for exertion of miR-7 anticancer regulatory effects against papillary carcinoma. The Bcl-2 targeting was also reported for miR-7 in lung cancer [26]. The in vivo mice study additionally supported the anti-cancer regulatory potential of miR-7 to be used in molecular anti-cancer strategy against the human papillary carcinoma.

Conclusion

Taken together, the study reveals miR-7 as an important molecular regulator of normal cell functioning and suggests that the decrease in expression of miR-7 is one the molecular events behind growth and development of human papillary carcinoma. The study also points towards the therapeutic implications of miR-7 in the treatment of human papillary carcinoma.

Disclosure of conflict of interest

None.

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References


Figure 5. miR-7 inhibited in vivo tumor development in mice. A. Tumors, excised from mice belonging group injected with miR-NC or miR-7 mimics TPC-1 cells along, with their respective weight in grams. B. Comparison of in vivo tumor weight (g). C. Comparison of in vivo tumor size (mm³). D. IHC of Ki67 miR-NC and miR-7 mimics xenografted tumor sections. E. IHC of cleaved caspase-3 in miR-NC and miR-7 mimics xenografted tumor sections. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).
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