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Original Article

Inhibition of papillary thyroid carcinoma growth by miR-200c via suppressing XIAP expression

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Abstract: Papillary thyroid carcinoma (PTC) is a common malignant tumor of thyroid tissues that affects people's health. The occurrence of PTC has been found to be related with abnormal expression of microRNA (miR). MiR-200c is correlated with occurrence of multiple tumors but has not been studied in PTC. This study thus investigated the correlation between miR-200c and PTC, in order to provide the evidence of screening PTC tumor markers. Real-time PCR was used to detect miR-200c expression in PTC and adjacent tissues, along with those in Nthy-ori3-1, TPC-2, and BCPAP cells. CCK8 assay was applied to detect the effect of miR-200c on BCPAP cell proliferation via its over-expression. Dual-luciferase reporter assay and Western blotting were used to elucidate the regulatory effect of miR-200c on X-linked inhibitor of apoptosis (XIAP). Expression level of miR-200c in PTC tissues was significantly lower than that in adjacent tissues ($t=4.84$, $P<0.001$). MiR-200c level in TPC-1 and BCPAP cells was obviously lower than that in Nthy-ori3-1 cells ($P<0.05$). MiR-200c transfection also decreased cell numbers ($P<0.05$). Luciferase intensity in miR-200c mimics + pmirGLO-XIAP-3'UTR-wt co-transfected cells was decreased by 60.92% compared with control group ($P<0.05$). XIAP protein expression was also suppressed by miR-200c mimics transfection. PTC pathogenesis might be related with miR-200c down-regulation. MiR-200c may inhibit PTC growth via down-regulating XIAP expression.

Keywords: Papillary thyroid carcinoma, miR-200c, XIAP, cell proliferation

Introduction

Papillary thyroid carcinoma (PTC) is a common malignant tumor of thyroid tissue [1, 2]. As the most common pathological subtype, it occupies about 90% of all cases [3]. In recent years, the incidence of thyroid cancer, especially PTC, increases rapidly [4]. Due to similar clinical symptoms as those of benign thyroid disease, PTC owns a high rate of misdiagnosis, leading to the miss of best treatment window, which is critical for improving patient's survival rate and quality of life. PTC lesion is insensitive to radio- or chemo-therapy, resulting in surgery as its primary treatment strategy. Therefore, the establishment of effective biological marker is important for the treatment of PTC.

MicroRNA (miRNA) is a group of small RNA fragments that participates in body growth, development, and disease occurrence. It is about 19~24 nt length as a non-coding RNA in eukaryotic cells. Via complete or incomplete complementary binding to 3' untranslated region (UTR) of target gene(s), miRNA can inhibit or degrade target mRNA at post-transcriptional level, thus mediating gene expression and is involved in the progression of diseases, including tumor, cardio-cerebro-vascular disease, and immune disorders. Meanwhile, the highly conserved sequence of miRNA attracts wide interests to study as potential biomarkers. Large amounts of studies showed the important role of abnormal miRNA expression in occurrence and progression of PTC. For example, miR-375 can inhibit cell proliferation and induce cell apoptosis via targeted regulating the expression of ERBB2 mRNA and protein [5]. The expression level of miR-31 is significantly decreased in PTC tissues. *In vitro* study showed that miR-31 overexpression could inhibit cell proliferation and invasiveness [6]. MiR-663 could also inhibit

tumor invasion and metastasis by regulating the expression of TGFβ1 [7].

As a member of miR-200 family, miR-200c has close correlation with colorectal carcinoma [8], prostate cancer [9], thymic lymphoma [10], and breast cancer [11]. Its relationship with PTC, however, has not been reported. This study thus investigated the expression level of miR-200c in pathological tissue of PTC and cell lines, in order to elucidate their correlation. Moreover, to further investigate the functional mechanism of miR-200c on PTC, we screened a candidate target gene of miR-200c, X-linked inhibitor of apoptosis (XIAP), via bioinformatics prediction. This study aimed to provide evidence for early diagnosis and targeted treatment of PTC.

Materials and methods

Reagents and equipment

Nthy-ori3-1, TPC-1 and BCPA cell lines (ATCC, US); DMEM culture medium (Gibco, US); CCK8 assay kit (Toyobo, Japan); Fetal bovine serum (Gibco, US); RNA extraction kit Trizol (Invitrogen Life, US); Reverse transcription kit (TaKaRa, Japan); Real-time fluorescent quantitative RT-PCR kit (TaKaRa, Japan). Gel imaging system and ViiA7 fluorescent quantitative PCR cycler (ABI, US); Total protein extraction kit (BestBio, China); Coomassie brilliant blue (Meiji Biotech, China); SDS-PAGE, PBST buffer, electrophoresis apparatus and GIS-2020D gel imaging analysis system (Sigma, US); XIAP and GAPDH antibody (Abcam, US); MiR-200c mimics and miR-200c mimics NC (Invitrogen Life, US); Lipofectamine 2000 (Invitrogen Life, US); pmirGLO-basic plasmid (Promega, US); Dual-Luciferase Reporter assay system (Promega, US); All primers were designed and synthesized by Dongyangfang (China).

Samples collection

A total of 20 cases of PTC and adjacent tissue samples were collected from an oncology hospital in Guangzhou, China from January 2014 to June 2015. All patients were diagnosed as PTC. Adjacent tissues were sampled from at least 2 cm beyond tumor edges. All tissues were frozen at -80°C for further use. In all 20 patients, there were 13 males and 7 females, with average age at 60.12±5.65 years. No patients received radio-, chemo-therapy, 131-I, or thyroid stimulating hormone (TSH) treatment before the surgery, nor did any other malignant tumors.

Cell culture and transfection

Nthy-ori3-1, TPC-1 and BCPAP cells were cultured in DMEM medium containing 15% fetal bovine serum (FBS) in a humidified chamber with 5% CO_2 at 37°C. Medium was changed every two or three generations. Cells were passaged when reaching 80%~90% confluence. After washing with PBS, cells were digested in trypsin to prepare suspensions and 200 ml cell suspensions were extracted for further cultivation. BCPAP cells were transfected with miR-200c mimics or miR-200c NC by Lipofectamine 2000 according to the expression level of miR-200c in cell lines following manual instruction. Firstly, 250 μl DMEM were used to dilute 5 μl Lipofectamine, meanwhile, equal volume of medium was applied to dilute 7.5 μl miR-200c mimics or NC. These two reagents were then mixed at room temperature for 20min to prepare miR-200c mimics/NC-Lipofectamine complex, which was then added into the medium. After 24 h incubation, the transfection rate was observed under a microscope. Those transfected cells with higher than 80% transfection efficiency were enrolled for further experiments, in which miR-200c or miR-Ctrl groups were included.

Real time PCR

Trizol reagent was used to extract total RNA from all cells and tissue samples. Purity and concentration of RNA was determined by nucleic acid analyzer. The integrity of RNA molecules was defined by 1% agarose gel electrophoresis. 1 μg RNA was used to synthesize cDNA based on the instruction of reverse transcription kit. Real-time PCR was performed in a system containing 4.5 μl 2XSYBR Green Mixture, 1 μl cDNA, 1 μl primer 1 (5 μM), 1 μl primer 2 (5 μM), and 2.5 μl ddH_2O. PCR conditions were: 95°C denature for 30 s, followed by 40 cyclers containing 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Each experiment was carried out in triplicates, with U6 as the internal reference gene.

CCK8 assay for cell proliferation

At 24 h, 48 h, and 72 h after transfection with miR-200c or miR-Ctrl, BCPAP cells along with

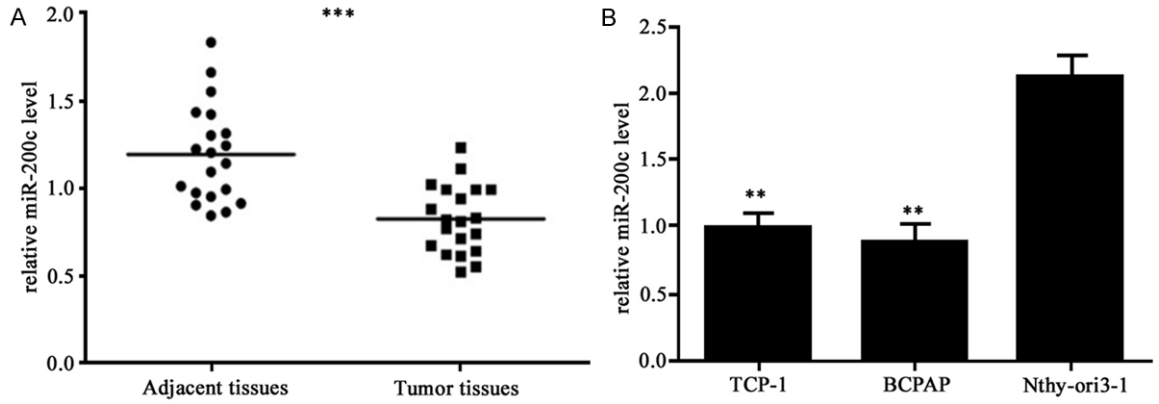

Figure 1. Expression levels of miR-200c in tumor tissues (A) and in related cell lines (B). Tumor tissues and adjacent tissues were collected from patients with PTC for extraction of total RNA, which was used for measuring the mRNA expression of miR-200c by real-time PCR (A). Meanwhile, total RNA was extracted from TCP-1, BAPAP or Nthy-ori3-1 cell lines for detection of the expression of miR-200c by real-time PCR (B). ***, P<0.001. **, P<0.01 compared with Nthy-ori3-1.

untransfected controls were assayed in CCK8 kit to determine cell proliferation ability (N=5 in each group). In brief, culture medium was washed away and discarded. After PBS rinsing for three times, 100 μL CCK8 mixture (1:10 dilution in culture medium) was added into each well for 2.5 h incubation at 37°C avoid of light. Absorbance values at 450 nm were measured in a microplate reader.

Dual-luciferase reporter gene assay

We further predict the target gene of miR-200c based on bioinformatics online tools. The candidate target gene XIAP was chosen based on complementary base pairing. 3'UTR region of XIAP gene was amplified, purified, digested, and ligated into pmirGLO eukaryotic expression vector containing luciferase gene to generate pmirGLO-XIPA-3'UTR-wt recombinant plasmid. Using similar methodology, XIAP-wt-miR-200c mimics, XIAP-wt-miR-200c NC, XIAP-mut-miR-200c mimics and XIAP-mut-miR-200c NC plasmids were co-transfected into BCPAP cells. After 24 h, the activity of Renilla and Firefly luciferase was detected by this system and was expressed as the relative activity of each pair of luciferase. Each experiment was repeated for three times.

Western blotting

MiR-200c, miR-Ctrl and control BCPAP cells were homogenized after 24 h of transfection. Total proteins were extracted and centrifuged to obtain the supernatant, in which protein content was quantified by Coomassie brilliant blue reagent. Before loading, protein samples were denatured by boiling. SDS-PAGE was used to separate protein samples, which were then transferred onto the NC membrane by electrical approach. The membrane was firstly blocked by PBST containing 5% defatted milk powder for 2 h at room temperature. After rinsing in PBST for three times, primary antibody was added for overnight incubation at 4°C. The membrane was rinsed for 30 min in PBST, and was incubated in secondary antibody dilutions containing 2.5% defatted milk powder for 60 min incubation. ECL reagent was then applied onto the membrane after PBST rinsing. The membrane was exposed in a dark room, and then was developed, rinsed and quenched. GIS-2020D gel imaging analyzing system was used to detect optical density of XIAP and β-actin protein bands. The ratio was then calculated to reflect the relative expression intensity of XIAP.

Statistical method

SPSS 13.0 software was used to analyze all collected data, and normally distributed data were presented as mean ± standard deviation. Between-group-comparison was performed by t-test. Statistical significance was defined as P<0.05.

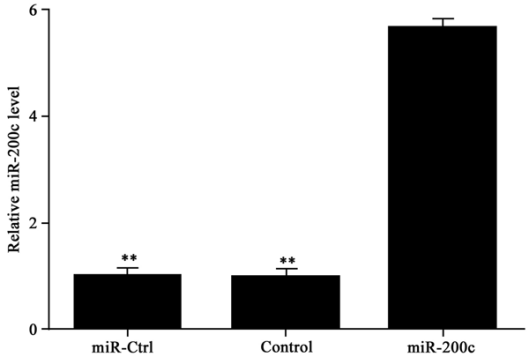

Figure 2. MiR-200c expression after mimics transfection in BCPAP cells. BCPAP cells were cultured in DMEM medium in a humidified chamber with 5% CO_2 at 37°C followed by passaging when reaching 80%~90% confluence. After washing with PBS and digestion in trypsin to prepare suspensions, BCPAP cells were transfected with miR-200c mimics or miR-200c NC by Lipofectamine 2000 according to manufacturer's instructions followed by measuring the expression of miR-200c by real-time PCR. **, P<0.01 compared to miR-200c group.

Table 1. BCPAP cell proliferation activity

Group	24 h	48 h	72 h
Control	0.65±0.03	0.81±0.05	0.98±0.06
miR-Ctr	0.63±0.04	0.83±0.03	0.96±0.05
miR-200	0.51±0.03[a,b]	0.61±0.04[a,b]	0.71±0.04[a,b]

Note: [a]P<0.05 compared to control group; [b]P<0.05 compared to miR-Ctrl group.

Results

Decreased expression of miR-200c in PTC and adjacent tissues

We used real-time PCR to detect the expressional profile of miR-200c in PTC tissues and tumor adjacent tissues. As shown in **Figure 1A**, miR-200c relative expression level in PTC tissues was significantly lower than that in adjacent tissues (0.83±0.19 vs. 1.20±0.28, t=4.84, P<0.001).

Reduced expression of miR-200c in PTC cell lines

Two PTC cell lines, TPC-1 and BCPAP, along with one normal thyroid cell line Nthy-ori3-1 were applied for detecting miR-200c expression intensity. As shown in **Figure 1B**, expression level of miR-200c in TPC-1 (1.00±0.11) and BCPAP (0.89±0.13) was significantly lower than that in Nthy-ori3-1 cell line (2.13±0.16, P<0.01).

Increased miR-200c expression level after transfection

In order to substantiate the effect of miR-200c mimics transfection, we firstly quantified miR-200c expression level in all groups of cells by real-time PCR. As shown in **Figure 2**, those BCPAP cells transfected with miR-200c mimics had significantly higher expression level as compared to miR-200c NC transfected group or untransfected controls (5.68±0.18 vs. 1.00±0.14 or 0.98±0.13, P<0.01). No significant difference had been found between miR-Ctrl and control group (P>0.05). Results showed elevated miR-200c expression in BCPAP cells after transfection, providing grounds for further assays.

Decreased proliferation activity of BCPAP cells induced by miR-200c

CCK8 assay was then employed to determine the effect of miR-200c on cell proliferation activity of all cells. Optical density (OD) values were shown in **Table 1**. After 24, 48 and 72 hours of transfection, cells transfected with miR-200c mimics had significantly decreased proliferation activity compared to miR-Ctrl group or control group (P<0.05), suggesting inhibition effect of miR-200c on cell proliferation. No significant difference can be found between miR-Ctrl group and control group (P>0.05).

Negative regulation of XIAP expression by miR-200c

Those abovementioned experiments showed the inhibition on BCPAP cell proliferation by miR-200c. To further elucidate its possible mechanism, we detect the targeted regulation on XIAP gene by miR-200c using a dual luciferase reported gene system. As shown in **Figure 3**, the relative luciferase signal intensity of miR-200c mimics + pmirGLO-XIPA-3'UTR-wt co-transfected group was decreased by 60.92% compared to miR-200c NC + pmirGLO-XIPA-3'UTR-wt co-transfected group (0.34 vs. 0.87 m P<0.05). In mutant pmirGLO-XIAP-3'UTR-mut, the level of miR-200c did not affect signal intensity of luciferase (P>0.05).

Meanwhile, we also tested the protein expression of XIAP in miR-200c, miR-Ctrl, and control groups. As shown in **Figure 4**, relative level of

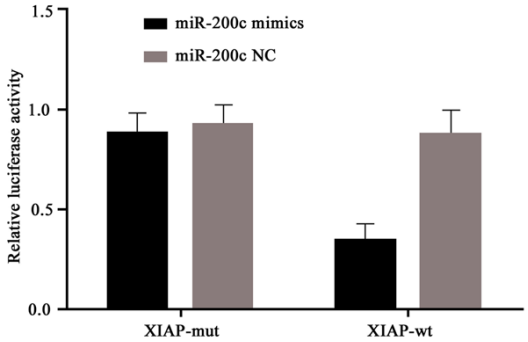

Figure 3. Relative luciferase activity. 3'UTR region of XIAP gene was ligated into pmirGLO eukaryotic expression vector containing luciferase gene to generate pmirGLO-XIPA-3'UTR-wt recombinant plasmid. Similarly, XIAP-wt-miR-200c mimics, XIAP-wt-miR-200c NC, XIAP-mut-miR-200c mimics and XIAP-mut-miR-200c NC plasmids were co-transfected into BCPAP cells. After 24 h, the activity of Renilla and Firefly luciferase was detected and was expressed as the relative activity of each pair of luciferase. Each experiment was repeated for three times. *, P<0.05 compared to miR-200c mimics + XIAP-wt co-transfected cells.

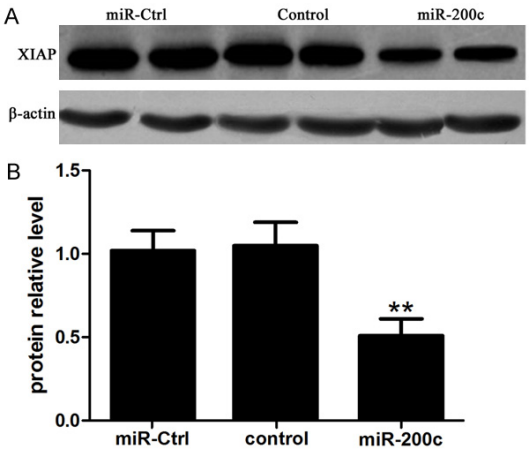

Figure 4. XIAP protein expression level. MiR-200c, miR-Ctrl and control BCPAP cells were homogenized after 24 h of transfection followed by extraction of total proteins which were loaded into SDS-PAGE for separation. Then the separated proteins were transferred onto the NC membrane and blocked by PBST followed by addition of anti-XIAP antibody HRP-conjugated secondary antibody. At last, ECL reagent was applied onto the membrane and then was developed, rinsed and quenched. β-actin was used as a control (A). The ratio was then calculated to reflect the relative expression intensity of XIAP (B). **, P<0.05 compared to miR-Ctrl and control group.

XIAP protein was significantly lower in miR-200c group as compared to miR-Ctrl group or control group (0.51±0.10 vs. 1.02±0.12 or 1.05±0.14, P<0.05). No significant difference was found between miR-Ctrl group and control group (P>0.05).

Discussion

Since it was discovered, miRNA has aroused wide interest in research due to its modulatory function on post-transcriptional regulation of gene expression. Meanwhile, its potency as a disease biomarker for diagnosis and treatment also draws lots of attention [12]. Piling data showed close correlation between abnormal expression of miRNA and various types of tumors such as pulmonary carcinoma [13], breast cancer [14], PTC [15], and kidney cancer [16]. Multiple miR molecules have been found to play important roles in PTC, such as miR-29a, which is downregulated in PTC [17]. MiR-9 and miR-21 can also be treated as markers for recurrence of PTC [18]. The up-regulation of miR-146a and miR-146b is closely correlated with clinical symptoms of PTC and can function as potent markers for PTC [19]. Current studies showed the close relationship between abnormal expression of miR-200c and occurrence of multiple tumors [9, 10]. However, no study had mentioned miR-200c in PTC. This study thus investigated the functional mechanism of miR-200c in PTC.

In a total of 20 PTC patients, miR-200c expression level in PTC samples was significantly suppressed compared with that in tumor adjacent tissues by real-time PCR, suggesting the correlation between PTC pathogenesis and miR-200c down-regulation. As miRNA exerts its function via targeted regulation on gene expression, further *in vitro* assay was performed to study its mechanism. Firstly, bioinformatics approach was used to screen target gene for miR-200c. Such relationship was then substantiated by dual-luciferase reporter gene system. Results showed significantly inhibited luciferase signal intensity in cells co-transfected with miR-200c mimics and pmirGLO-XIAP-3'UTR-wt. After up-regulation of miR-200c, XIAP protein expression level was also significantly down-regulated, suggesting close targeted relationship between miR-200c and XIAP whose expression can be inhibited by miR-200c at post-transcriptional level. XIAP is a known critical molecule inducing tumor cell apoptosis

[20]; its expression was thus closely correlated with tumor cell apoptosis. Study found high expression of XIAP in PTC tissues, suggesting its potency as a marker for evaluating both treatment efficacy and prognosis of PTC [21]. MiR-200c was down-regulated in PTC. As results of this study showed down-regulation of XIAP by miR-200c, it is highly likely that miR-200c might lose the inhibition of XIAP expression, thus inducing the up-regulation of XIAP in PTC for further tumor occurrence. Similar studies also found the inhibition of cell proliferation and induction of cell apoptosis by down-regulating XIAP in triple-negative breast cancer [22].

In summary, this is the first time that the correlation between miR-200c and PTC was studied. Our results showed the possible correlation between low-expression of miR-200c and PTC pathogenesis, as miR-200c might inhibit PTC cell growth via down-regulating XIAP. Whether miR-200c induces PTC occurrence by inhibition of XIAP after its down-regulation in population, however, require further substantiation.

Disclosure of conflict of interest

None.

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