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Original Article

MiR-34a targeted Notch2 to induce apoptosis of medullary thyroid carcinoma cells

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Abstract: No effective strategy other than surgery can treat medullary thyroid carcinoma (MTC). Notch1 expression can induce apoptosis of MTC cells. This study aimed to investigate the correlation between miR-34a and Notch1 in MTC, plus their role in apoptosis of MTC cells. qRT-PCR was firstly used to test expression profiles of miR-34a in MTC tissues. Bioinformatics and luciferase reporter gene were employed to determine targeted gene of miR-34a. TT cell line was transfected by miR-34a mimic. Western Blot was used to examine Notch1 expression, followed by flow cytometry for cell cycle and cell proliferation. qRT-PCR found significantly depressed miR-34a expression in MTC cells ($P<0.05$ compared to tumor adjacent tissues). Both bioinformatics analysis and luciferase reporter gene assay demonstrated Notch1 as potential target of miR-34a. MiR-34a mimic transfection into TT cells remarkably inhibited Notch1 expression level ($P<0.05$). Flow cytometry found that over-expression of miR-34a significantly decreased cell number at S phase ($P<0.05$), elongated cell cycle, decreased cell proliferation activity and elevated apoptosis level. MiR-34a was down-regulated in MTC cells. It can inhibit proliferation potency of MTC cells via targeted inhibition on Notch1 expression, thus facilitating cell apoptosis.

Keywords: MiR-34a, Notch1, medullary thyroid carcinoma, cell apoptosis

Introduction

Medullary thyroid carcinoma (MTC) is derived from calcitonin-secreted parathyroid follicular cells, and is mainly caused by RET gene mutation on chromosome 10. MTC has relatively higher malignancy and frequently develops metastasis via lymph tract or blood circulation. Once having metastasis of neck lymph node, it can rapidly infiltrate out of capsule to affect peripheral tissues [1, 2]. Currently, surgery is the major strategy for treating MTC. However, when cancer cells undergo metastasis, major difficulty exists for patient treatment [3]. Therefore, the regulation mechanism of behaviors of MTC cells is important for exploring novel treatment plans.

MiR-34a belongs to microRNA family, which is one group of non-coding small RNA molecule with 21-25 bp [4]. Previous study showed that microRNA could affect multiple cell behaviors via mediating target gene expression level [5]. Recent studies believed that miR-34a exerted its physiological role via affecting Notch1 signal pathway [6]. Notch1 signal pathway is widely distributed in all animal cells, and exerts important roles in regulating various animal cells, and is closely correlated with occurrence of multiple malignant tumors and cell regulation [7-9]. This study aimed to investigate the effect of miR-34a on Notch1 signal pathway and its regulatory role on MTC cell proliferation.

Materials and methods

Research subjects

A total of 20 MTC patients who were admitted in Suizhou Hospital, Hubei University Of Medicine from February 2015 to December 2015. All patients did not receive any chemo-or radio-therapy before admission. Pathology examination was performed to confirm MTC diagnosis. There were 9 males and 6 females, with average age = 42.1 ± 6.5 years. Tumor tissues and adjacent tissues were collected during the surgery, and were kept in liquid nitrogen for further mRNA assay. This study has been approved

Table 1. Primer sequences using in qRT-PCR

Name	Sequence	Tm (°C)
miR-34a-F	5'TGTGAGTGTTTCTTTGGCAGTG3'	55.7
miR-34a-R	5'ACAACGTGCAGCACTTCTAG3'	54.2
U6-F	5'CTCGCTTCGGCAGCACA3'	55.3
U6-R	5'AACGCTTCACGAATTTGCGT3'	55.4

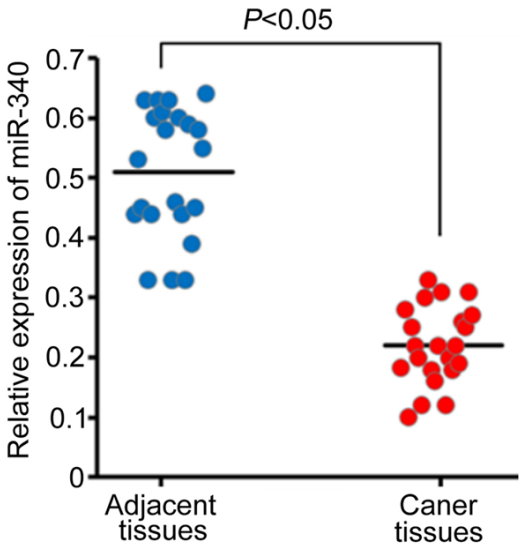

Figure 1. MiR-34a expression in MTC tumor and adjacent tissues.

by the ethical committee, and all participants have signed informed consents.

qRT-PCR

qRT-PCR was used to test expressional profile or miR-34a in MTC tissues and normal tissues. Firstly, primers were designed based on miR-34a sequence (GeneBank access number: NR_029610) as shown in **Table 1**. Total RNA was extracted from liquid nitrogen kept tissues by RNAprep pure Tissue Kit (Qiagen). Using total RNA from tumor adjacent tissues as the control group, miR-34a expression was determined by mirVanatqRT-PCR miRNA test kit (Ambion). PCR was performed for 40 cycles at 60°C annealing temperature. Built-in software V2.02 was used for analysis using U6-RNA as the internal reference by $2^{-\Delta\Delta Ct}$ method [10].

MiR-34a function prediction

TargetScan Release 5.1 (www.targetscan.org) bioinformatics software was used to predict miR-34a function. Luciferase reporter gene was used to confirm possible target sites of miR-344a. Based on mRNA sequence of Notch1 (Genebank access number: NM_017617), oligonucleotide sequence Notch1-3'-UTR was synthesized based on bp155-206 site of Notch1 3'-UTR. Sequence of Notch1-3'-UTR was: 5'-GTCAC TAGTA TGTAC TTTTA TTTTA CACAG AAACA CTGCC TTTTT ATTTA TATGT ACTGA AGCTT CTC-3'. Notch1-3'UTR sequence was inserted downstream of firefly luciferase gene coding region in pmirGLO vector to construct pmirGLO-notch1 plasmid. Using MTC cell line TT as the research subject, transfection of pmirGLO-notch1 and pmirGLO vectors were separately performed. Those cells with transfection were transfected by miR-34a mimic to elevate miR-34a activity (as described in section 1.5). Analysis of fluorescent intensity was performed 48 h after successful transfection. Dual luciferase reporter gene analysis system (Promega) was used for analysis of fluorescent intensity on MicroLumatPlus LB96V spectrometry (Berthold).

TT cell transfection

Based on miR-34a sequence, miR-34a mimic and miR-34a inhibitor were designed and synthesized by Sangon (China). INTERFERinTM transfection kit (Polyplus transfection) was used for cell transfection by liposome at 50 nM concentration. MTC cell line TT was purchased from Cell Bank of Chinese Academy of Science. Frozen cells were firstly resuscitated and digested by trypsin, and were counted for inoculation into 96-well plate using freshly prepared medium. 24 h after incubation, transfection was performed using liposome INTERFERinTM transfection kit (Polyplus transfection) following the manual instruction of test kit [11].

Western blot

TT cells at log-growth phase were collected and lysed in 100 μL lysis buffer for culture and centrifugation. The supernatant was extracted for protein component, which was analyzed by Western Blot. After electrophoresis, transfer, blocking and primary antibody immune reaction (mouse anti-human Notch1 or β-actin antibody). HRP-labelled secondary antibody (goat anti-mouse IgG) was then added, followed by DAB development. Western Blot results were captured by computer and were analyzed for integrated gray values of bands by gel imaging

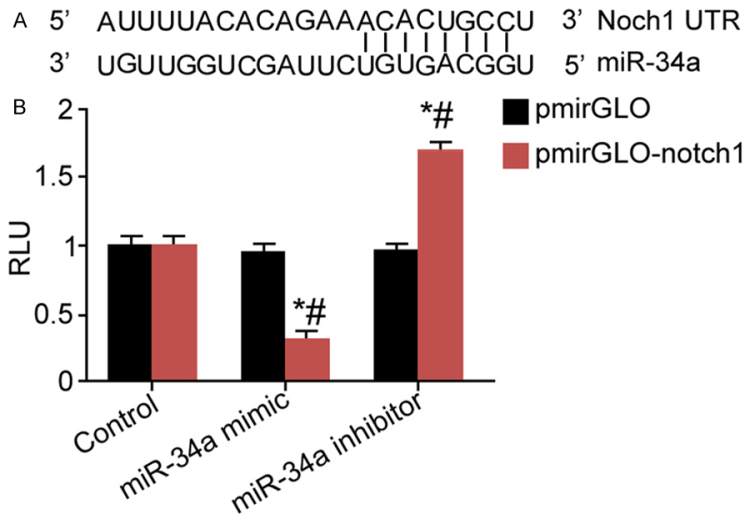

Figure 2. Notch1 gene and miR-34a. A. Homologous analysis between 3'UTR of Notch1 and miR-34. B. Luciferase reporter gene assay results. *, P<0.05 compared to control group; #, P<0.05 compared to pmir-GLO vector in the same group.

system. Using β-actin as the internal reference, relative expression level of Notch1 protein expression in samples was calculated [12].

Flow cytometry analysis

Flow cytometry was used to test proliferation ability of TT MTC cells after transfection of miR-34a mimic or inhibitor. Using untreated TT cell as the control group, cells were removed from culture medium and were rinsed twice in PBS. By fixation in 90% ethanol overnight, RNAse was used for digestion. After PI staining, flow cytometry (Becton Dickinson) was used for flow cytometry analysis at 488 nm excitation wavelength and 630 nm emission wavelength. 10000 fluorescent signals were collected from each cell sample in triplicates. Modifit software was used to analyze FL-2 area and DNA histogram of samples to reveal cell cycle distribution [13].

Statistical analysis

SPSS 20.0 software was used for statistical analysis. Results were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used for comparing between-group difference. Student t-test was used for comparison. A statistical significance was defined when P<0.05. Extreme significance was defined when P<0.01.

Results

qRT-PCR results

qRT-PCR was used to test miR-34a expression in MTC tumor tissues and adjacent tissues. MiR-34a relative expression level was analyzed as shown in **Figure 1**. By calculating relative expression level of miR-34a in tumor and adjacent tissues in different MTC patients, we found significantly lowered miR-34a relative expression level in MTC tumor tissues (P<0.05 compared to tumor adjacent tissues). By calculating miR-34a expression level in thyroid tissues from different origins, miR-34a relative expression level was 0.22, as decreased by 56.9% compared to tumor adjacent tissues (0.51, P<0.05).

Bioinformatics prediction for miR-34a target

TargetScan Release 5.1 (www.targetscan.org) bioinformatics software was used to predict function of miR-34a. Results indicated Notch1 as the potential target of miR-34a. By sequence homologous analysis (**Figure 2**), we found certain homology between miR-34a and 3'UTR of Notch1 mRNA sequences. Luciferase reporter gene assay found significantly lowered cell fluorescent intensity in cells after miR-34a mimic transfection (P<0.05). Whilst the transfection of miR-34a inhibitor in MTC TT cells remarkably increased fluorescent intensity (P<0.05). These results suggested that miR-34a function on 3'UTR of Notch1 gene.

Notch1 protein expression

By Western Blot method, we analyzed Notch1 protein expression in MTC cells. As shown in **Figure 3**, in those TT cells with successful miR-34a mimic transfection, Notch1 protein expression was significantly down-regulated (P<0.05). Whilst in those TT cells with successful transfection of miR-34a inhibitor, Notch1 protein expression was up-regulated (P<0.05). These results suggested that over-expression of miR-34a in MTC TT cells decreased Notch1 protein expression.

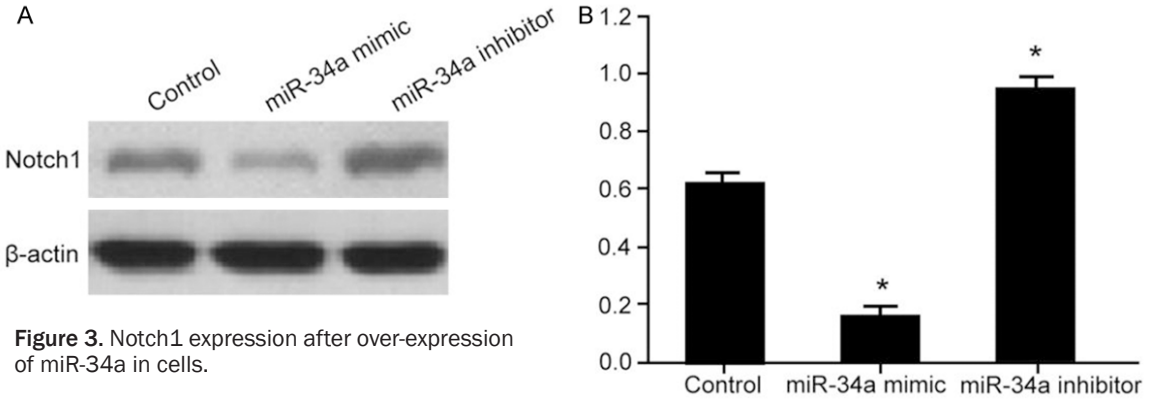

Figure 3. Notch1 expression after over-expression of miR-34a in cells.

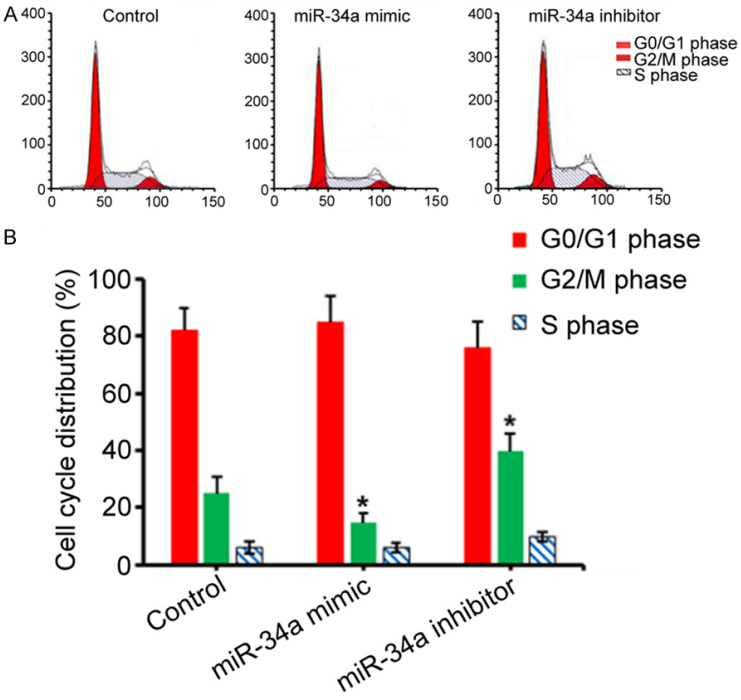

Figure 4. Flow cytometry for cell cycle.*, P<0.05 compared to control group.

Flow cytometry for cell proliferation potency

Flow cytometry was used to test proliferation potency of TT MTC cells with miR-34a mimic or miR-34a inhibitor transfection. Modifit software was used to analyze FL-2 area and DNA histogram as shown in **Figure 4**. By counting cell number at different cycles, we found significantly decreased cell number at S phase in TT cells with miR-34a mimic transfection (P<0.05 compared to control group), whilst cell number at G0/G1 phase and G2/M phase all remained indifferent (P>0.05). These results showed that TT cells with miR-34a mimic transfection had elongated cell cycle and weakened proliferation potency. On the other hand, miR-34a inhibitor transfection had increased cell number at S phase (P<0.05), whilst no significant change in cell number at G0/G1 at G2/M phase (P>0.05). These results showed that miR-34a inhibitor transfection shortened TT cell cycle and enhanced proliferation potency.

Discussion

To study the expression of miR-34a in MTC, and it regulatory role on tumor cell proliferation via targeting Notch1, we firstly performed RT-PCR, which found significantly decreased miR-34a expression in MTC patients. We then performed bioinformatics analysis and found that Notch1 was potential target of miR-34a. To further explore the regulation of miR-34a on MTC cell proliferation via targeting Notch1, we used MTC cell line TT as the model, on which cell transfection was performed to enhance or suppress miR-34a expression, followed by cell proliferation assay. Results showed down-regulation of Notch1 expression in TT cells with miR-34a over-expression, accompanied with elongated cell cycle and weakened proliferation potency. The inhibition of miR-34a, on the other hand, potentiated TT cell proliferation. In one word, miR-34a can inhibit Notch1 protein expression in MTC cells via targeting its gene, thus further suppressing cell proliferation and potentiating apoptosis.

Various studies demonstrated major difference in microRNA expression profile between human malignant tumor cells including MTC cells and normal cells [14-16]. Recent study attributed miR-34a as the functioning target of tumor suppressor gene P53, which can arrest mitosis and facilitate apoptosis [17]. Long et al found significantly decreased miR-34a expression in MTC cells compared to normal tissues [18]. The mechanism of how MTC cells down-regulate miR-34a expression is still debatable now. Some studies believed that abnormal methylation of promoter CpG island for miR-34a expression led to down-regulation of miR-34a [19]. This conclusion, however, still requires more evidences for supporting.

Belonging to microRNA family, miR-34a can regulate cell behavior via RNA interference mechanism. Li et al performed bioinformatics analysis and luciferase reporter gene assay, and demonstrated that miR-34a could inhibit Notch1 expression level at transcription level via binding onto 3'UTR of Notch1 mRNA [20]. Notch signal pathway is one evolutionary conserved, and affecting cell differentiation, development and death regulation. Adjacent cells can transmit Notch signal via binding between Notch1 receptor and ligand, thus amplifying molecular difference between cells, eventually determine cell fate and affecting organ formation or morphogenesis [6]. Current study revealed the close correlation between Notch1 with some malignant tumors including breast cancer and prostate cancer [7, 9]. Notch1 exerts its function mainly via affecting cell cycle. Recent study revealed dual roles of Notch1 on cancer cells as both oncogene and tumor suppressor gene [21, 22].

Early diagnosis and treatment of MTC has become a major challenge for clinicians [2]. This study demonstrated the relationship between miR-34a expressional profile of MTC cells and Notch1. This feature can work as evidences for clinical diagnosis, and may provide insights for treating MTC using RNA interference in future.

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Disclosure of conflict of interest

None.

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