

## Original Article

# Expression of miR-34a and its role in human papillary thyroid carcinoma

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**Abstract:** Objective: To investigate the biological function of microRNA-34a (miR-34a) in human papillary thyroid carcinoma (PTC). Methods: 89 pairs of human PTC and adjacent normal tissue were obtained. Real-time PCR were used to determine the level of miR-34a. Correlation of miR-34a with development of PTC was also analyzed. BCPAP and TPC1 cells were transfected with miR-34a mimics and scrambled miRNA was set as negative control. The effects of miR-34a on cell proliferation and cell cycle were evaluated by CCK8 and flow cytometry, respectively. Results: The relative expression level of miR-34a was significantly higher in PTC tissues than that in corresponding adjacent non-tumorous thyroid tissues; however, we found that relative lower expression level of miR-34a was observed in PTC with unfavorable features, including large tumor size, multi-focal distribution, lymph metastasis and high TNM stages. CCK8 assay showed that cell proliferation was inhibited after transfection of miR-34a mimics. MiR-34a could block cell cycle transition from G0/G1 phase into S phase in TPC1 cells. Conclusion: MiR-34a was up-regulated in PTC, and relative lower level of miR-34a was related to unfavorable features of PTC. It inhibits proliferation of PTC cells and block cell cycle transition from G0/G1 phase to S phase. MiR-34a acts as a tumor suppressor gene in PTC.

**Keywords:** Papillary thyroid carcinoma, miRNA-34, cell cycle, human

### Introduction

MicroRNAs (miRNAs), small noncoding RNAs of approximately 22 nucleotides, suppress post-transcriptional expression of target genes through binding to the 3'-untranslated region (3'-UTR) [1, 2]. miRNAs play important roles in fundamental biological processes such as cell proliferation, differentiation and apoptosis [3]. For many decades, dysregulation of miRNAs has also been reported in various types of human cancers [4, 5], suggesting it may play a crucial role in the development of human cancer.

Thyroid carcinoma is one of the most common endocrine-related cancers globally. Among the four types of thyroid carcinoma (papillary, follicular, medullary and anaplastic), papillary thyroid carcinoma (PTC) is most common, accounting for 80% of all cases. Various expression profiles of miRNAs are reported to be involved in development of different types of thyroid car-

cinoma. The expression level of *miR-211*, *miR-222* and *miR-146* was found to be up-regulated [6] in PTC using miRNA microarray analysis; *miR-197*, *miR-187* and *miR-183* levels were elevated in follicular thyroid carcinoma (FTC) [1]; *miR-222*, *miR-198*, *let-7f-1* and *let-7a-2* were up-regulated in anaplastic thyroid carcinoma (ATC) [3].

*MiR-34a* was first discovered in *Caenorhabditis elegans* and belongs to the evolutionarily conserved miRNA34 family [7]. A growing number of researches have shown that miR-34a acts as a tumor mimics by suppressing various genes which promote cell proliferation [8-10]. It is directly regulated by classic tumor suppressor gene *p53* [11] and is often down-regulated in various types of human cancers, such as lung cancer [12], pancreatic cancer [13], colorectal cancer [14], bladder cancer [10, 15, 16] and breast cancer [17, 18]. However, there are still exceptions. In human PTC tissues and cell lines, *miR-34a* has been reported to be up-regulated

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**Table 1.** Correlation of miR-34a with clinical pathological data

Clinical parameters	N	Relative miR-34a level <sup>a</sup>	p value
Age (year)			0.355
≥50	45	0.364 (0.267-0.491)	
<50	44	0.351 (0.273-0.468)	
Sex			0.624
Male	31	0.381 (0.221-0.451)	
Female	58	0.354 (0.320-0.481)	
Tumor size (cm)			0.032*
≥2	49	0.310 (0.230-0.440)	
<2	40	0.420 (0.330-0.591)	
Distribution			0.029*
Sigle-focal	41	0.450 (0.370-0.535)	
Multi-focal	48	0.302 (0.250-0.390)	
Bilateral PTC			0.671
Yes	24	0.375 (0.235-0.460)	
No	65	0.381 (0.287-0.481)	
Capsular invasion			0.210
Yes	32	0.393 (0.312-0.410)	
No	57	0.362 (0.281-0.475)	
Lymphatic metastasis			0.047*
Yes	51	0.321 (0.280-0.451)	
No	38	0.412 (0.372-0.548)	
Tumor stages <sup>b</sup>			0.044*
III-IV	30	0.310 (0.287-0.420)	
I-II	59	0.442 (0.312-0.556)	

\* $P < 0.05$ , with significant difference; <sup>a</sup>relative expression level of miR-34a, expressed as median with interquartile range; <sup>b</sup>TNM stage was referred to the sixth edition of 2002 AJCC cancer staging manual.

[19-21]. Ma et al. proposed that miR-34a might have a pro-tumor effect in PTC, and their results showed that miR-34a could promote cell proliferation and inhibit apoptosis [21], suggesting miR-34a might act as an oncogene in PTC. It's quite interesting for a specific miRNA to function oppositely in different types of human cancers. However, the expression and role of miR-34a in PTC have been less reported up to now. Based on our previous results of miRNA microarrays in human PTC tissues, we aim to study the level of *miR-34a* in different stages of human PTC tissues, and further investigate its role in cell proliferation.

### Materials and methods

#### Human tissues

89 patients with PTC (male, 31 cases) were treated in the Department of Surgical Oncology

in Taizhou Municipal Hospital (Taizhou, Zhejiang, China). 89 pairs of human PTC and adjacent normal tissue were obtained from these patients. Excised human tissues were immediately snap-frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$ . Informed consent was obtained from all patients. Basic information for the patients was shown in **Table 1**. The median age of male patients was 51 years (range: 33-78 years); the median age of female patients was 49 years, ranging from 18-71 years. TNM stage was referred to the sixth edition of 2002 AJCC cancer staging manual. None of the patients had a history of neck radiation. Thyroid cancer was diagnosed by preoperative ultrasonography and puncture. Further diagnose of PTC was made pathologically after surgery. All patients underwent total thyroidectomy with neck lymph node dissection. Lymph node metastasis was found in 46 patients.

#### Total RNA extraction and real-time PCR

Total RNA from PTC tumor and adjacent tissues was extracted using TRIzol reagent according to manufacturer's instructions. The concentration of total RNA was detected using a spectrophotometer. The integrity of RNA was detected by 1% denaturing agarose gel electrophoresis. Then, total RNA was reverse-transcribed using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Otsu, Shiga, Japan)

for miR-34a expression. Real-time PCR was carried out using SYBR green Real-time PCR Master Mix on an ABI 7500 Real-time PCR instrument (Applied Biosystems). The conditions of real-time PCR were as following:  $95^{\circ}\text{C}$  for 30 s;  $95^{\circ}\text{C}$  for 5 s,  $55^{\circ}\text{C}$  for 10 s to anneal, and  $72^{\circ}\text{C}$  for 15 s to elongate followed by 40 cycles. U6 was used as internal control. Relative gene expression levels were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method.

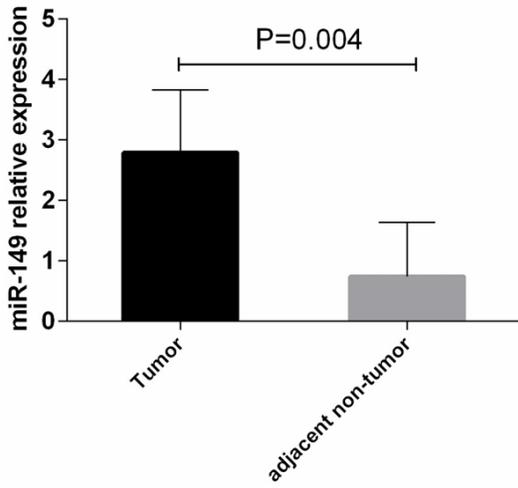
#### Cell culture

Human PTC cell line BCPAP and TPC1 were purchased from the ATCC (Massachusetts, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  in a humidified incubator.

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**Table 2.** Sequences for miR34a mimics and scrambled miRNA

Name	Sequence
MiR-34a	
Sense	5'-UGAGAUGAAGCACUGUAGCUC-3'
Anti-sense	5'-UUACUCUACUUCGUGACAUCG-3
Scrambled miRNA	
Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
Anti-sense	5'-TTAAGAGGCUUGCACAGUGCA-3



**Figure 1.** MiR-34a is up-regulated in human PTC tissues. Relative expression level of miR-34a detected by real-time PCR in PTC tissues and their adjacent nontumorous counterparts.

## Oligonucleotide transfection

BCPAP and TPC1 cells were seeded in 6-well plate and cultured in DMEM supplemented with 10% FBS without antibiotics. When 80% confluence was reached, the cultured cells were transfected with miR-34a mimics or miRNA scrambled (negative control, NC) using Lipofectamine 2000. The sequences of miR-34a mimics and negative control were shown in **Table 2**.

## Cell viability assay

Cells were seeded into 96-well plates at a density of  $3 \times 10^3$  per well. After 12 hours, miR-34a mimics or NC was transfected into the cells according to the above-mentioned method. At 24, 48 and 72 hours post-transfection, 20  $\mu$ l of CCK8 was added into each well and cells were incubated for an hour at 37°C. The optical density at 450 nm was detected on a microplate reader.

## Flow cytometry assay

Cells were seeded into 6-well plates at a density of  $3 \times 10^5$  per well. MiR-34a mimics or NC were transfected according to the above-mentioned method after 12 hours. Cells were collected 48 hours post-transfection. The transfected cells were fixed in 70% cold ethanol overnight at 4°C, and then stained in propidium iodide for one hour at 4°C in the dark. The fluorescence was read on a Flow Cytometer (BD, USA), and the percentage of cells at each stage of cell cycle was analyzed by Flowjo software.

## Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS, Chicago, IL). Data with normal distribution was expressed as mean  $\pm$  SD (standard deviation), and Statistical significance was assessed by comparing mean values ( $\pm$  SD) using the two-tailed Student's t-test for two independent groups or one-way ANOVA followed by LSD post-hoc test. Data with abnormal distribution was expressed as median (IQR, interquartile range). The expression level of miR-34a in clinical PTC tissues and their adjacent nontumorous counterparts was compared using a non-parametric Wilcoxon signed-rank test. Correlation of the level of miR-34a with basic information and clinical pathological data was analyzed by Mann-Whitney U test. The probability value  $P < 0.05$  was considered to be statistically significant.

## Results

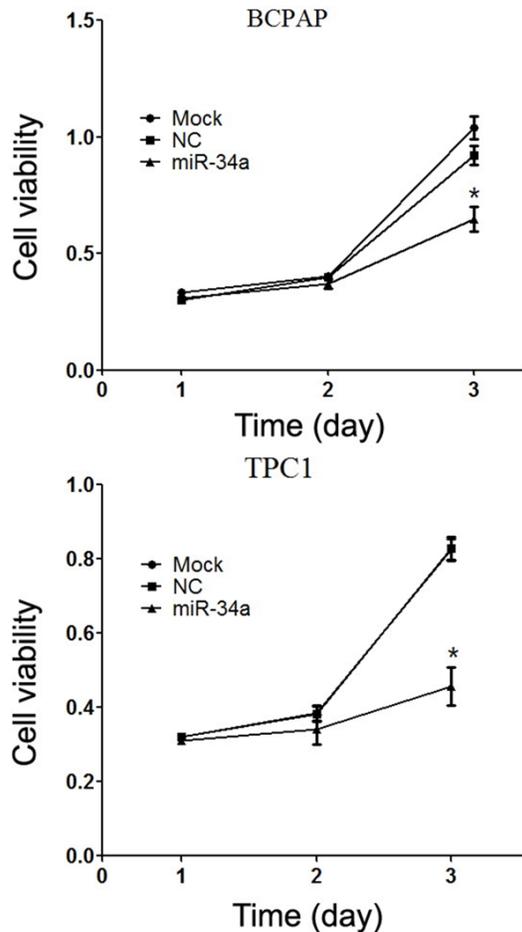
### MiR-34a is up-regulated in human PTC tissues

The results of real-time PCR showed that the relative expression level of miR-34a was significantly higher in PTC tissues than that in corresponding adjacent nontumorous thyroid tissues (**Figure 1**), the median expression level in the tumor tissues was almost 3.3 times higher than correspondent adjacent nontumorous tissues.

### Expression level of miR-34a and clinical features of PTC

Correlation of basic information and clinical pathological data with the expression level of miR-34a was analyzed by Mann-Whitney U test. Our results showed that relative lower expression level of miR-34a was observed in PTC with

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**Figure 2.** MiR-34a inhibits proliferation of thyroid cancer cells. The cell viability was detected by MTS assay in BCPAP and TPC1 cells.

unfavorable features, including large tumor size, multi-focal distribution, lymphatic metastasis and high TNM stages (**Table 1**). No significant correlation was found between the expression level of miR-34a and sex, age, bilateral PTC and capsular invasion (**Table 1**). Our results suggested that the relative lower level of miR-34a might be related to PTC with unfavorable features, and lower level of miR-34a might exert tumor inhibition function in PTC.

### *MiR-34a transfection inhibits proliferation of thyroid cancer cells*

The results of CCK8 assay showed that cell proliferation was inhibited after transfection of miR-34 mimics. Significant difference was found at day 3 post-transfection ( $P < 0.01$ ) (**Figure 2**) and the inhibitory rate in BCPAP and TPC1 cells was 20.1% and 39.6%, respectively

(**Figure 2**). Our results suggest that miR-34a inhibits proliferation of thyroid cancer cells.

### *Effect of miR-34a on cell cycle*

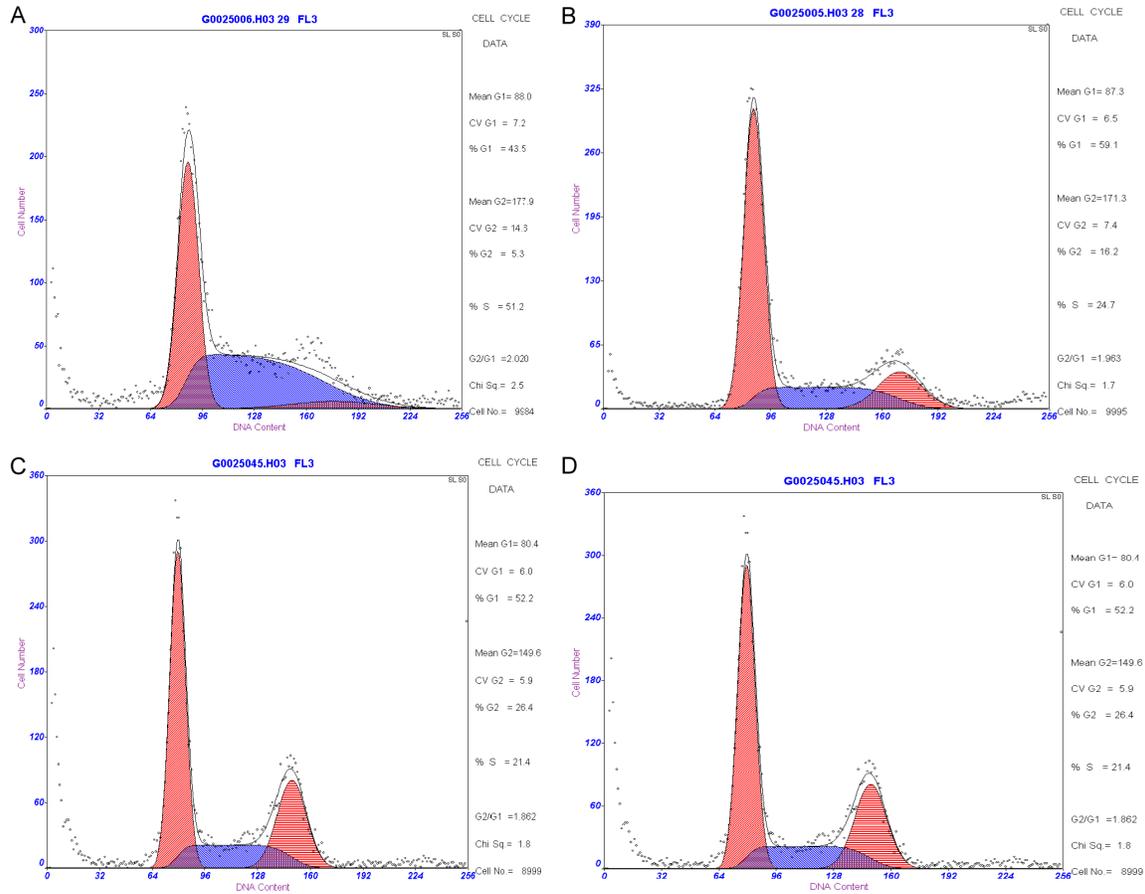
BCPAP and TPC1 cells were transfected with miR-34a mimics and scrambled miRNA was set as negative control. After 48 hours, the distribution of cell cycle was analyzed by flow cytometry analysis. Our results showed that the percentage of BCPAP cells at G0/G1 was 24.32% after transfection with scrambled miRNA, while the percentage was 52.29% after transfection with miR-34a mimics (**Figure 3A** and **3B**). On the other hand, the percentage of TPC1 cells at G0/G1 was 29.68% when transfected with scrambled miRNA, while the percentage was 51.22% when transfected with miR-34a mimics (**Figure 3C** and **3D**). Our results suggest that miR-34a could inhibit cell cycle transition from G0/G1 phase into S phase in these two PTC cells.

### **Discussion**

MicroRNAs have been drawn great attention in recent years in diagnose, treatment and prognosis of thyroid carcinoma. Increasing evidence have revealed that dysregulation of miR-34a are involved in various types of cancer. MiR-34a plays important roles in fundamental biological processes, such as cell proliferation, differentiation and apoptosis [3, 22-24]. Tarasov et al. reported that miR-34a could induce apoptosis and cell cycle arrest in the G1 phase, thereby suppressing tumor cell proliferation [23]. Tazawa et al. revealed that abrogation of miR-34a function could contribute to aberrant cell proliferation, leading to the development of colon cancer [25]. The level of miR-34a is down-regulated in many types of cancer, and it may work as a tumor suppressor through inhibiting cell proliferation, promote cell apoptosis and inhibits cell metastasis [9, 15, 16, 22]. However, in human PTC tissues and cell lines, the level of miR-34a was found to be up-regulated [19-21].

Ma et al. proposed that miR-34a might have a pro-tumor effect in PTC, and their results showed that miR-34a could promote cell proliferation and inhibit apoptosis [21], suggesting miR-34a might act as an oncogene in PTC. It's quite interesting for a specific miRNA to function oppositely in a particular type of cancer,

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**Figure 3.** Effect of miR-34a on cell cycle. BCPAP and TPC1 cells were transfected with miR-34a mimics and scrambled miRNA was set as negative control. After 48 hours, the distribution of cell cycle was analyzed by flow cytometry analysis. The percentage of BCPAP cells at different cell cycle phase transfected with NC (A) or miR-34a (B); the percentage of TPC1 cell at different cell cycle phase transfected with NC (C) or miR-34a (D).

compared with its function in other type of human cancer. Previously, we detected the miRNA expression profile in PTC and normal thyroid tissues using high throughput microarrays. We found that the expression level of miR-34a was significantly up-regulated for more than 3.6 times in PTC tissues compared with normal thyroid tissues. In this study, we confirmed the up-regulation of miR-34a in PTC tissues by using real-time PCR.

We also analyzed the standard clinicopathological data, such as tumor size, tumor TNM stages, tumor invasion, and lymphatic metastasis. Interestingly, we found that the relative low level of siR-34a was observed in PTC with unfavorable features, including large tumor size, multi-focal distribution, lymph metastasis and high TNM stages. Our results showed that though the level of miR-34a was up-regulated

in PTC tissues, the relative low level of miR-34a was related to unfavorable features of PTC. Based on these results, we proposed that miR-34a may not act as a main pro-tumor factor, though it was up-regulated in PTC. It may be a protective factor but passively up-regulated by BRAF mutation [26], DNA damage or ectopic p53 [27, 28].

To further elucidate the role of miR-34a in the development of PTC, human PTC BCPAP and TPC1 cells were transfected with miR-34a. Our results showed that miR-34a should significantly inhibit tumor cell proliferation both in BCPAP and TPC1 cells. Moreover, miR-34a could suppress cell cycle transition from G0/G1 phase into S phase in BHP cells. These results suggest that miR-34a could inhibit proliferation of PTC cells through suppressing cell cycle tra-

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sition from G0/G1 phase into S phase. MiR-34a may also act as a tumor suppressor in PTC, which is similar to its function in other human cancers [5, 8, 29-31]. A growing number of literature suggest that miR-34a could simultaneously modulate multiple signaling pathways [4, 32, 33]. Mir-34a has been reported to down-regulate the level of E2F3, which is a key regulator in the cell cycle transition from G1 phase to S phase [25]. It also has potential to abrogate the effect mediated by the dysfunction of p53-Rb signaling pathway. Multiple targets of miR-34 within different pathways make it involved in various basic biological processes, including suppressing cell cycle progression. Further study is needed to clarify the underlying mechanism of miR34 on cell cycle transition in PTC.

In conclusion, miR-34a was up-regulated in PTC, but relative low level of miR-34a was related to unfavorable features of PTC. It could inhibit proliferation of PTC cells and suppress cell cycle transition from G0/G1 phase to S phase. MiR-34a acts as a tumor suppressor in PTC.

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### Disclosure of conflict of interest

None.

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