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Original Article

Downregulation of miR-381 is associated with poor prognosis in papillary thyroid carcinoma

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Abstract: Circulating microRNAs (miRNAs) are potential biomarkers for papillary thyroid carcinoma (PTC). The aim of this study was to evaluate the diagnostic and prognostic value of serum miR-381 in PTC. A total of 87 patients with PTC, 50 cases with benign thyroid nodules (BTN) and 50 healthy volunteers were enrolled. The expression levels of serum miR-381 were measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results indicated that serum miR-381 expression was significantly decreased in PTC patients compared to that of BTN patients or healthy controls. Moreover, serum miR-381 showed good performance to differentiate PTC cases from controls. Next, reduced serum miR-381 expression was positively correlated with aggressive clinical features and shorter overall survival. Furthermore, serum miR-381 levels were greatly elevated in 21 patients with advanced-stage (stage III/IV) PTC after surgery. Finally, univariate and multivariate Cox regression analysis confirmed that miR-381 in serum was an independent prognostic indicator for OS in PTC patients. Collectively, serum miR-381 might serve as a non-invasive biomarker for the diagnosis and prognosis of PTC.

Keywords: Papillary thyroid carcinoma, biomarker, diagnosis, prognosis, MiR-381

Introduction

Thyroid cancer is the most frequent type of endocrine-related cancer, and its incidence and prevalence is steadily rising around the world in the past decades. Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid, which accounts for 85%-90% of all thyroid cancer [1, 2]. Although PTC is a relatively indolent and highly curable disease, about 10% of the patients would eventually die of this disease [3, 4]. Therefore, it is important to identify novel disease biomarkers for stratifying the PTC patients and making tailored therapeutic options accordingly.

MicroRNAs (miRs) are small, highly conserved non-coding RNA molecules with 18-25 nucleotides in length that negatively regulate gene expression at a post-transcriptional level [5]. MiRNAs downregulate the specific target genes either through translational repression or direct degradation. These small molecules have been demonstrated to involve in many biological processes such as proliferation, differentiation, survival and apoptosis. Therefore, deregulated expression of miRNAs is closely linked to the many human diseases including cancer [6, 7]. There is an increasing amount of evidence that miRNAs play a critical role in the carcinogenesis of PTC, either act as tumor growth promoters or suppressors [8, 9].

miR-381 is located within a 20 kb genomic region (cluster-14-miRNAs) on chromosome 14q32.31 [10]. Aberrant expression of miR-381 is implicated in various types of cancers, suggesting that miR-381 might exert either oncogenic or tumor-suppressive functions. Some studies show that miR-381 acts as a tumor suppressor in hepatocellular carcinoma [13], colorectal cancer [14], epithelial ovarian cancer

Table 1. Expression of serum miR-381 in subgroups divided by clinical characteristics in PTC patients

Characteristics	Number of patients	Low miR-381 (n=42)	High miR-381 (n=45)	*P*-value
Gender				0.1543
Male	18	6	12	
Female	69	36	33	
Age				0.0937
<45	50	28	22	
≥45	37	14	23	
Tumor size				0.0820
≤1.0 cm	28	9	19	
1.0-2.0 cm	35	18	17	
≥2.0 cm	24	15	9	
Lymph node metastasis				0.0252*
No	46	17	29	
Yes	41	25	16	
Extrathyroidal invasion				0.0009*
No	43	13	30	
Yes	44	29	15	
Tumor multifocality				0.6123
No	62	31	31	
Yes	25	11	14	
TNM stage				0.0006*
I, II	66	25	41	
III, IV	21	17	4	
Tumor bilaterality				0.2318
No	67	30	37	
Yes	20	12	8	

*P value <0.05.

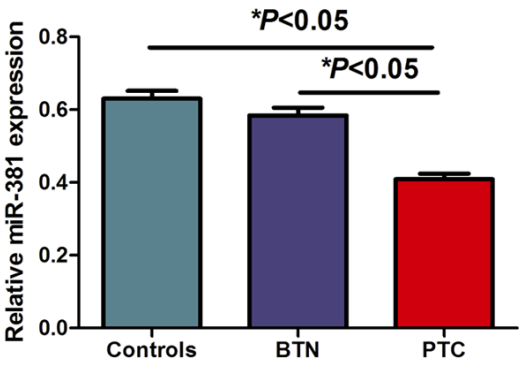

Figure 1. Serum miR-381 levels differ significantly between the PTC group and the BTN or control groups.

[15], lung adenocarcinoma [16], colon cancer [17], breast cancer [18], gastric cancer [19] and pituitary tumor [20]. On the contrary, some reports suggest that miR-381 exhibits oncogenic property in osteosarcoma [21] and glioma [22]. However, the expression pattern and clinical significance of miR-381 in PTC remains unclear. The aim of current study was to investigate the potential prognostic value of miR-381 in PTC.

Materials and methods

Study population

The study protocol was approved by the Ethics Committee of Drum Tower Clinical Medical College of Nanjing Medical University and written informed consent was obtained from all participants. Eighty-seven PTC patients who scheduled to receive surgical resection, and 50 patients with benign thyroid nodules (BTN) were enrolled in this study. Of the 87 cases of PTC, 62 cases of classical variant, 16 cases of follicular variant, and 9 cases of tall cell variant. Among 50 BTN subjects, 16 subjects showed a classical nodular goiter and 34 subjects had a thyroid adenoma. Moreover, 50 healthy volunteers were recruited as controls. The cancer stage was determined based on the 7th edition AJCC TNM staging system. Detailed information of the PTC cases were summarized in **Table 1**.

Serum samples and RNA extraction

Venous blood (approximately 5 mL) was taken from all participants prior to any treatment. Within 1 hour after collection, all samples were processed and centrifuged at 3000 g for 15 min. Subsequently, the separated supernatant was aliquoted into cryotubes and stored at -80°C until RNA extraction.

Total RNA was isolated from blood samples using the miRVana PARIS kit (Ambion, Austin, TX, USA). For normalization of sample-to-sam-

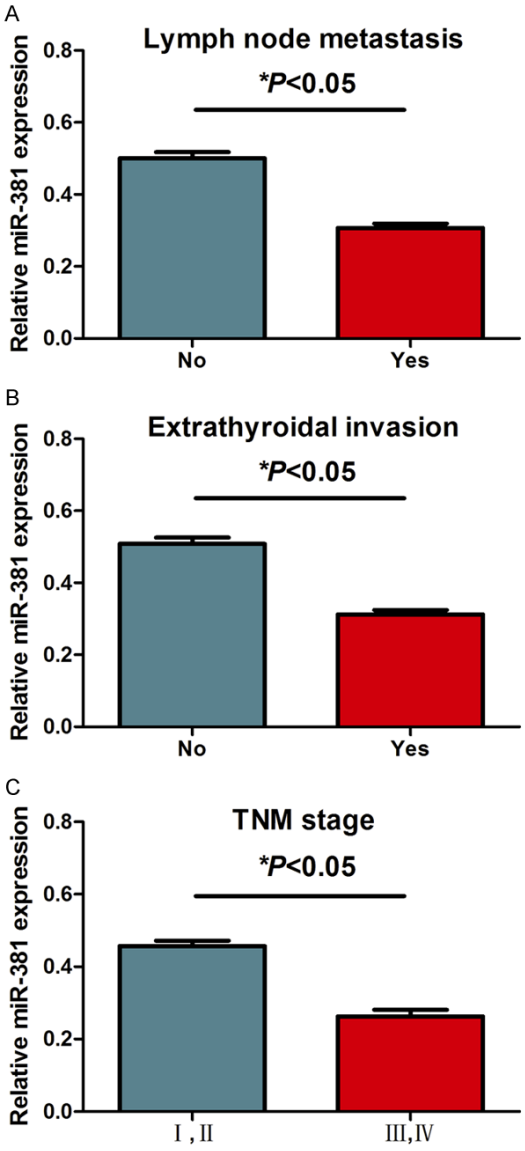

Figure 2. A. Serum miR-381 levels in PTC patients with lymph node metastasis were decreased compared to those without. B. Serum miR-381 levels in PTC patients with extrathyroidal invasion were decreased compared to those without. C. Serum miR-381 levels in PTC patients with higher TNM stage were lower than those with lower TNM stage.

ple variation, 25 fmol of synthetic C. elegans miRNA cel-miR-39 (Qiagen, Mississauga, ON, Canada) was added to each denatured sample. The RNA concentration and quality was determined on a Nanodrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

Quantitative reverse-transcription polymerase chain reaction

2 μg total RNA was used in the reverse transcription reaction and first strand cDNA was synthesized using a Reverse Transcription kit (Takara, Dalian, China). Quantitative real-time PCR analysis was analyzed with SYBR® Green PCR Master Mix (Takara) on a 7500 Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. All experiments were conducted in triplicate and the relative serum miR-381 expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the PCR primers were as follows: miR-381 forward, 5'-AGTCTATACAAGGGCAAGCTCTC-3', miR-381 reverse, 5'-ATCCATGACAGATCCCTACCG-3'.

Statistical analysis

Statistical analysis was performed with MedCalc 17.4 (MedCalc Software, Ostend, Belgium) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney U test or Kruskal-Wallis test was used to compare the difference of miR-381 expression between groups. Chi-square test was used to compare miR-381 levels and various clinicopathological parameters of PTC. Receiver operating characteristic (ROC) curve and the area under the curve (AUC) was performed to analyze the diagnostic value of serum miR-381 expression. The overall survival (OS) curves were calculated using the Kaplan-Meier method with log-rank test. The correlations between risk factors and OS were evaluated by univariate and multivariate Cox proportional hazard analysis. A *p*-value less than 0.05 was considered to be statistically significant.

Results

Serum miR-381 was significantly reduced in PTC patients and ROC analysis

The present study used qRT-PCR to determine the expression level of serum miR-381 from all participants. The results indicated that serum miR-381 levels in PTC patients were significantly lower than those in BTN subjects and controls, while there was no significant difference between BTN patients and healthy controls (**Figure 1**). In addition, serum miR-381 expression was markedly downregulated in PTC cases with lymph node metastasis compared to those without ($P<0.05$, **Figure 2A**). Also, PTC patients with extrathyroidal invasion had a lower serum miR-381 level than that of those without ($P<0.05$, **Figure 2B**). Moreover, serum miR-381 expression levels were dramatically increased in patients with early-stage PTC compared to

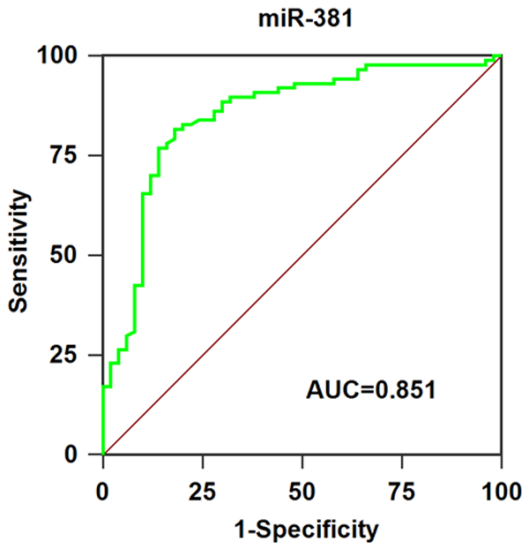

Figure 3. ROC analysis using miR-381 for distinguishing PTC patients from controls.

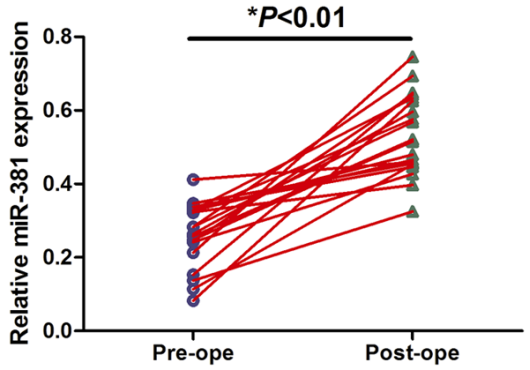

Figure 4. Levels of serum miR-381 in 21 patients with stage III/IV PTC were greatly upregulated one month after surgery.

those with advanced-stage disease ($P<0.05$, **Figure 2C**).

Next, ROC analysis was processed to evaluate the diagnostic value of serum miR-381. We found that serum miR-381 had an AUC of 0.851, with 81.6% sensitivity and 82.0% specificity (**Figure 3**). The results suggested that serum miR-381 could well differentiate PTC subjects from normal controls.

Correlation of serum miR-381 and clinicopathological characteristics of PTC

To determine the correlation of serum miR-381 in PTC subjects with clinical features, all 87 PTC patients were classified into two groups based on the median serum miR-381 expression level. The low expression group had 42 cases and the high expression group had 45 cases. As shown in **Table 1**, serum miR-381 expression was closely associated with lymph node metastasis ($P=0.0252$), extrathyroidal invasion ($P=0.0009$) and TNM stage ($P=0.0006$). However, no significant differences were found between serum miR-381 expression with gender ($P=0.1543$), age ($P=0.0937$), tumor size ($P=0.0820$), tumor multifocality ($P=0.6123$) and tumor bilaterality ($P=0.2318$).

Dynamic monitoring of serum miR-381 of PTC patients before and after surgery

To investigate the alteration of serum miR-381 levels in PTC patients, we obtained plasma samples from 21 PTC cases of stage III/IV for qRT-PCR analysis one month after surgery. Compared to preoperative blood specimen, miR-381 expression levels in postoperative plasma samples were greatly increased ($P<0.01$, **Figure 4**).

Correlation of serum miR-381 and prognosis in PTC patients

Figure 5A showed that the PTC cases in high serum miR-381 expression group had longer OS than those in low expression group ($P=0.042$). Additionally, Kaplan-Meier curves were constructed to assess OS for all PTC patients based on several clinical variables. No significant relationship was found between OS and lymph node metastasis (**Figure 5B**, $P=0.057$), and extrathyroidal invasion (**Figure 5C**, $P=0.061$). Whereas, patients with higher TNM stage was strongly associated with worse OS (**Figure 5D**, $P=0.018$).

As shown in **Table 2**, univariate analysis demonstrated that extrathyroidal invasion (HR=5.53, 95% CI=2.89-8.27, $P=0.017$), serum miR-381 (HR=4.76, 95% CI=2.28-7.29, $P=0.039$) and TNM stage (HR=6.63, 95% CI=3.42-10.15, $P<0.001$) were significant prognostic factors. Moreover, multivariate analysis identified lymph node metastasis (HR=4.88, 95% CI=2.35-7.50, $P=0.035$), extrathyroidal invasion (HR=6.06, 95% CI=3.12-9.16, $P=0.009$), serum miR-381 (HR=5.14, 95% CI=2.53-7.91, $P=0.026$) and TNM stage (HR=7.58, 95% CI=3.74-11.62, $P<0.001$) as independent risk factors for OS in PTC patients.

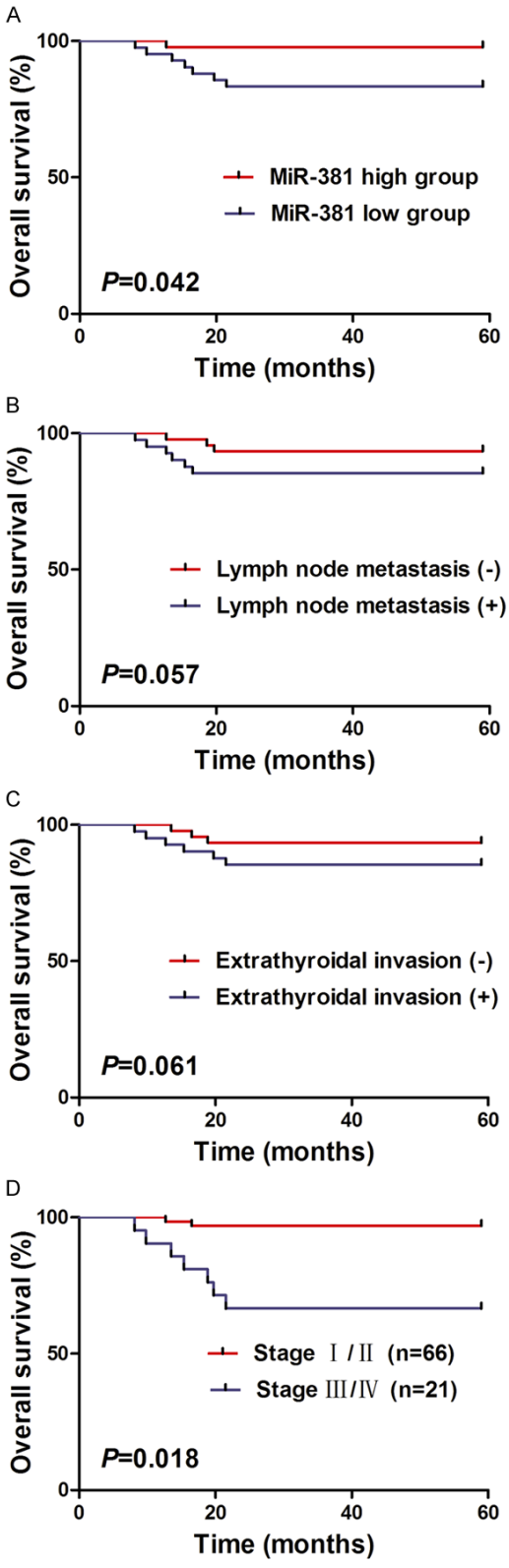

Figure 5. A. PTC patients in high serum miR-381 expression group had longer OS. B. The association between OS and lymph node metastasis. C. The association between OS and extrathyroidal invasion. D. PTC patients in advanced stage (III/IV) had poorer OS.

Discussion

PTC is an endocrine disease and has one of the highest familial risk ratios of all cancer types [11, 12]. To the best of our knowledge, the present study was the first report regarding the role of miR-381 in PTC. In this study, we found that serum miR-381 expression was significantly downregulated in PTC patients compared to that of BTN patients or healthy controls. PTC cases with lower miR-381 expression experienced more frequent lymph node metastasis, extrathyroidal invasion, and were correlated with advanced clinical stage. Moreover, ROC curve analysis proved that serum miR-381 could effectively differentiate PTC subjects from normal controls. Furthermore, decrease serum miR-381 expression was positively associated with aggressive clinical characteristics and shorter OS. Next, serum miR-381 levels in blood samples from 21 patients with stage III/IV PTC were markedly increased after treatment. Also, serum miR-381 expression was identified to be an independent prognostic indicator for PTC. All these findings strongly suggested that miR-381 might exert a tumor-suppressing role in PTC.

MiR-381 had been reported to play a tumor suppressive role in several cancers. For instance, miR-381 expression was greatly reduced both in hepatocellular carcinoma tissues and cell lines. Upregualtion of miR-381 significantly suppressed hepatocellular carcinoma tumorigenic potential by targeting liver receptor homolog-1 [13]. In colorectal cancer, decreased miR-381 expression was observed in cancerous tissues and closely associated with poor clinical variables. Inhibition of miR-381 markedly promoted cancer cell invasion, migration and epithelial-mesenchymal transition through regulating Twist1 [14]. In epithelial ovarian cancer, Xia et al reported that miR-381 was dramatically downregulated in cancer tissues and cell lines. Moreover, restoration of miR-381 reduced tumorigenicity by degrading YY1 [15]. In lung adenocarcinoma, a reduction in miR-381 expression was found in cancer tissues and correlated with shorter survival. Enhanced miR-381 expression suppressed carcinogenesis *via* inversely regulating ID1 [16]. Liang and others revealed that miR-381 expression was decreased in colon cancer tissues. MiR-381 overexpression attenuated cellular proliferation *in vitro* and tumor growth of nude mice *in*

Table 2. Univariate and multivariate analyses of parameters predictive of overall survival in 87 PTC patients

Parameters	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Gender, female *vs.* male	1.05 (0.83-1.27)	0.452	1.24 (0.95-1.58)	0.374
Age, <45 *vs.* ≥45	1.18 (0.92-1.45)	0.416	1.33 (1.02-1.72)	0.361
Tumor size, ≥2 *vs.* <2	2.25 (1.31-3.26)	0.276	2.56 (1.40-3.82)	0.243
Lymph node metastasis, yes *vs.* no	4.52 (2.17-7.14)	0.058	4.88 (2.35-7.50)	0.035*
Extrathyroidal invasion, yes *vs.* no	5.53 (2.89-8.27)	0.017*	6.06 (3.12-9.16)	0.009*
TNM stage, III/IV *vs.* I/II	6.63 (3.42-10.15)	<0.001*	7.58 (3.74-11.62)	<0.001*
MiR-381 in serum, low *vs.* high	4.76 (2.28-7.29)	0.039*	5.14 (2.53-7.91)	0.026*

**P* value <0.05; CI=confidence interval; HR=hazard ratio.

vivo [17]. In breast cancer, Xue and others demonstrated that miR-381 expression was dramatically downregulated in cancer tissues and cell lines. Additionally, ectopic miR-381 expression significantly decreased cancer cell proliferation, epithelial-to-mesenchymal transition and metastasis by silencing CXCR4 expression [18]. In gastric cancer, downregulation of miR-381 was found both in cancerous tissues and cell lines, and correlated with poor clinical characteristics and prognosis. MiR-381 inhibition promoted tumorigenesis *in vitro* and *in vivo* by regulating TMEM16A [19]. Liang et al found that miR-381 was greatly underexpressed in pituitary tumors and loss of miR-381 enhanced pituitary tumor cell tumorigenesis. Moreover, PTTG1 was identified as a downstream target of miR-381 [20].

Conversely, some previous studies had demonstrated that miR-381 played an oncogenic role in various types of cancer. In osteosarcoma, Li et al revealed that patients with high miR-381 expression had worse prognosis. Silencing miR-381 restrained tumor cell proliferation and invasion ability by directly regulating LRRC4, and improved the chemotherapeutic drugs sensitivity of osteosarcoma cells [21]. In glioma, Tang and others reported that forced miR-381 expression induced cancer cell proliferation *in vitro* and promoted tumor growth *in vivo*. Furthermore, LRRC4 was reported as a target gene of miR-381 [22]. Thus, the role of miR-381 in tumorigenesis appears to be very complex.

In conclusion, this study confirmed that serum miR-381 could serve as a promising marker for diagnosis and treatment monitoring for PTC patients. Nonetheless, the small sample size is a limitation of this current study. Thus, future analysis in a large cohort of PTC patients will be required.

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Disclosure of conflict of interest

None.

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