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Original Article

Serum miRNA-203 as a potential biomarker for papillary thyroid carcinoma

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Abstract: Objective: This study aimed to investigate the potential value of serum miR-203 as noninvasive recurrence-predictive and prognostic biomarker in patients with papillary thyroid carcinoma (PTC). Methods: 165 patients received total thyroidectomy in Ningbo No. 2 Hospital from June, 2008 to September, 2014 were enrolled in this study. Serum expressions of miR-203 were evaluated from the obtained blood samples from all the PTC patients by quantitative real-time PCR. The association between serum miR-203 expressions with the recurrence and overall survival rate in PTC patients were analyzed. Results: Serum miR-203 expressions were significantly increased in PTC patients with tumor recurrence in comparison with those with no recurrence (P<0.001). ROC analysis suggested that serum miR-203 expression was a significant predicative factor for PTC recurrence with an AUC of 0.755, a sensitivity of 71.1% and a specificity of 80.0% respectively. Results from Kaplan-Meier survival curves showed that high serum miR-203 serum expression was significantly associated with poor overall survival in PTC patients (log-rank P<0.001). The multivariate analysis with Cox regression analysis demonstrated that serum miR-203 expression was an independent prognosis factor for the overall survival rate of PTC patients (HR=6.75, 95% CI: 1.43-15.43, P=0.001). Conclusions: This study demonstrated the potential role of serum miR-203 expression as a non-invasive predicative biomarker for the recurrence and prognosis of PTC.

Keywords: Papillary thyroid carcinoma, serum miRNA-203, noninvasive biomarker, recurrence, prognosis

Introduction

Thyroid cancer is the worldwide prevalent endocrine neoplasm with almost 300,000 new cases and nearly 40,000 deaths per year [1]. Papillary thyroid carcinoma (PTC) is the most common histological type, accounting for 80-90% of all thyroid cancers and it is generally an indolent tumor with a low cancer-specific mortality [2]. During the patients who underwent total thyroidectomy, about 20-50% of them were with lymph node metastasis, and 5-20% might develop regional recurrence [3, 4]. The risk factors for the recurrence and overall survival of PTC patients were with no consensus in different studies or guidelines. The exact impact of neck node metastasis on the prognosis of PTC patients still remains controversial [5]. Early predication of PTC recurrence by noninvasive approaches was with significant clinical value. MicroRNAs (miRNAs) are small single-stranded, non-coding RNA strands (19-25 nucleotides in length) and they play important roles in the translation of specific protein coding genes in mammals [6]. MiRNAs are also known to be involved in the development, metastasis-related process in various types of cancers [7, 8]. Numerous studies have revealed that miRNAs play important roles in PTC prognosis [9, 10] and specific miRNAs are also associated with aggressive clinicopathologic features of PTC [11, 12]. Previous studies have reported that miR-203 expression is significantly up-regulated in PTC compared with benign nodular goiter and healthy controls [13, 14]. However, the correlation between PTC recurrence and prognosis with miR-203 expression levels remains unclear.

Material and methods

Patients and samples

This study was approved by the Medical Institutional Ethics Committee of Zhejiang province. 165 patients scheduled to undergo total

Table 1. Clinical and pathological characteristics of 165 PTC patients with or without recurrence

Parameters	Recurrence		*P*-value
	Yes (n=48)	No (n=117)	
Age (year)	45.8±14.3	48.7±17.1	0.302
Gender			
Male	12 (25.0%)	40 (34.2%)	
Female	36 (75.0%)	77 (65.8%)	0.249
ATA risk			
Low	12 (25.0%)	57 (48.7%)	
Intermediate	35 (72.9%)	59 (50.4%)	
High	1 (2.1%)	1 (0.9%)	0.018
MSKCC-NY risk			
Low	7 (14.6%)	35 (29.9%)	
Intermediate	22 (45.8%)	68 (58.1%)	
High	19 (39.6%)	14 (12.0%)	<0.01
Tumor size (cm)			
≥2	28 (58.3%)	40 (34.2%)	
<2	20 (41.7%)	77 (65.8%)	0.004
Cervical lymph node metastasis			
Yes	15 (31.3%)	20 (17.1%)	
No	33 (68.7%)	97 (82.9%)	0.043
TNM stage			
I-II	23 (47.9%)	87 (74.4%)	
III-IV	25 (52.1%)	30 (25.6%)	0.001
Vascular invasion			
Yes	26 (54.2%)	43 (36.8%)	
No	22 (45.8%)	74 (63.2%)	0.039
Perineural invasion			
Yes	26 (54.2%)	40 (34.2%)	
No	22 (45.8%)	77 (65.8%)	0.017
Extrathyroidal extension			
Yes	28 (58.3%)	44 (37.6%)	
No	20 (41.7%)	73 (62.4%)	0.015
Histological subtype			
Classic	37 (77.1%)	78 (66.7%)	
Follicular	10 (20.8%)	28 (23.9%)	
Classic and follicular	1 (2.1%)	11 (9.4%)	0.204
Relative expression of miR-203	4.31±1.65	2.85±1.27	<0.01

PTC, Papillary thyroid carcinoma; ATA, American Thyroid Association; MSKCC-NY, Memorial Sloan Kettering Cancer Center-New York.

thyroidectomy by the same surgical team in Ningbo No. 2 Hospital from June, 2008 to September, 2014 were enrolled in this study.

The detailed inclusion criteria were as follows: (1) histopathologic diagnosis with PTC after the surgery; (2) with no history of other cancers; (3) without history of preoperative treatment of radiotherapy or chemotherapy; (4) patients who can persist the follow-up; (5) provided the written informed consent. Patients with distant metastasis, incomplete clinical information or lack of follow-up data were excluded from this study. Patients included were categorized into two groups: the recurrent group and the non-recurrent group. Patients with no evidence of clinical, laboratorial and radiological recurrence or distant metastasis after a follow-up of 60-month were considered recurrence-free. Selective neck dissections were performed in patients with regional lymph node metastasis according to node metastases location. Patients in this study received the postoperative radioactive iodine (RAI) therapy under the guidance of the American Thyroid Association (ATA) [3]. In brief, the patients with primary tumor size >4 cm or extrathyroidal extension were submitted to postoperative RAI therapy. RAI therapy was also suggested for those with primary tumor size <4 cm who had documented intrathyroidal vascular invasion or lymph node metastases. The histopathologic features of PTC were all evaluated by two independent pathologists who were blinded to this study. PTC patients enrolled were staged according to Memorial Sloan Kettering Cancer Center-New York (MSKCC-NY) [15] and ATA risk stratification system and the Union for International Cancer Control-TNM classification [16].

Blood samples were all obtained from the 165 PTC patients on the day before the surgery. A5 ml of fast peripheral venous blood was drawn from all the patients and then it was placed at room temperature for 1 h. Then the collected samples were centrifuged (1000 g, 10 min, 4°C) to spin down the blood cells. All the collected serum samples were than stored at -80°C for further detection. The overall survival (OS) time was calculated

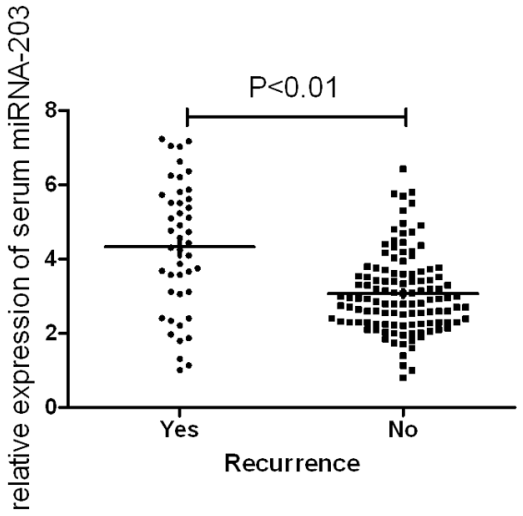

Figure 1. The serum miR-203 expressions and recurrence of PTC. Serum expressions of miR-203 were significantly elevated in PTC patients with recurrence compared to those without recurrence (P<0.01).

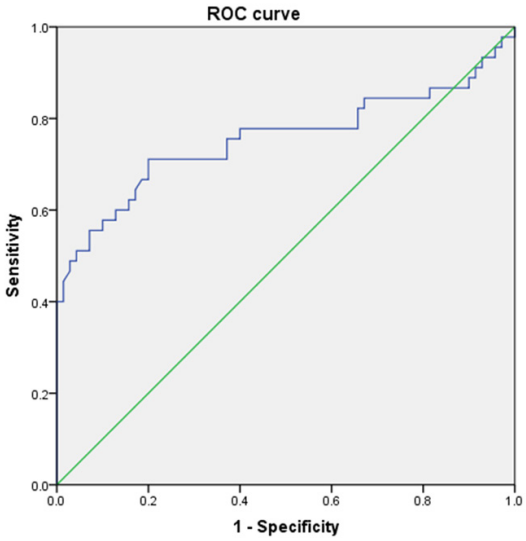

Figure 2. The analysis of predicative value of serum miR-203 for PTC recurrence by receiver operating characteristics (ROC) curve analysis. The areas under the ROC curve (AUC) was 0.755, with 95% confidence interval of 0.651 to 0.860 (P<0.001).

from the date of surgery to the death or the end of follow-up. The detailed clinical and pathologic characteristics of the PTC patients are shown in **Table 1**.

RNA isolation and quantitative real-time PCR

The miRNeasy™ RNA isolation kit (Qiagen, Valencia, CA, USA) was used for performing the isolation of miRNA from serum samples according to the manufacturer's instructions. Recover All Total Nucleic Acid Isolation Kits (Ambion, Austin, Texas, USA) were used for the performing miRNA extraction from samples. The miRNA expression was detected and quantified by using TaqMan miR real-time quantitative reverse-transcription PCR (qRT-PCR) (Applied Biosystems, Foster City, California, USA). The analysis of relative miRNA expression with GAPDHas endogenous controls by using $2^{-\Delta\Delta Ct}$ method according to the manufacturer's guidelines.

Statistical analysis

SPSS 21.0 (SPSS, Inc.) and GraphPad Prism 5 (GraphPad Software Inc., CA, USA) were used for statistical analysis in the study. Data are presented as number (n) and percentage (%) for categorical variables, or mean ± standard error (SD) as quantitative variables. Student's t-test was used for the evaluation of differences between groups. Fisher's exact or Chi-square test was used for the categorical comparisons. The correlations between risk factors and PTC recurrence were evaluated by univariate and multivariate Cox proportional hazard models. Survival analysis was evaluated by using Kaplan-Meier method, log-rank test and multivariate Cox regression analysis. The prediction of serum miR-203 for the recurrence of PTC was analyzed by using receiver operating characteristic (ROC) curve. All statistical tests were bilateral probability and P<0.05 was accepted as statistically significant.

Results

Serum expressions of miR-203 in PTC patients with or without recurrence

We detected the serum expressions of miR-203 by using the obtained blood samples from 165 PTC patients by the analysis of qRT-PCR and the result was shown in **Figure 1**. Serum expressions of miR-203 were significantly elevated in PTC patients with recurrence compared to those without recurrence (P<0.01), which suggested a potential predicative role of miR-203 in recurrence of PTC. Receiver operating characteristic (ROC) curve analysis was then utilized to evaluate the predicative value of miR-203 for PTC recurrence and we observed that areas under the ROC curve (AUC) was

Table 2. Univariate and multivariate analyses of factors with PTC recurrence by Cox regression analysis

	Univariate		Multivariate	
Parameter	HR (95% CI)	*P* value	HR (95% CI)	*P* value
ATA risk				
Intermediate x low	3.16 (1.07-7.89)	0.022*	0.81 (0.19-3.31)	0.58
MSKCC-NY risk				
Intermediate x low	0.89 (0.21-3.24)	0.78		
High x low	3.54 (1.09-10.91)	0.09		
High x Intermediate	1.78 (0.79-4.53)	0.17		
Tumor size				
≥2 cm x <2 cm	4.47 (2.01-13.33)	0.004*	1.43 (0.87-6.87)	0.40
Cervical lymph node metastasis	3.32 (0.79-5.98)	0.09		
TNM stage				
I/II x III/IV	3.98 (1.25-10.98)	0.003*	4.54 (1.10-9.98)	0.019*
Vascular invasion	2.24 (0.68-5.53)	0.10		
Perineural invasion	3.04 (0.87-7.75)	0.30		
Extrathyroidal extension	2.89 (1.10-8.85)	0.020*	1.53 (0.43-4.34)	0.35
Serum miR-203 level	1.38 (1.10-1.87)	0.001*	1.43 (1.15-1.85)	0.014*

PTC, Papillary thyroid carcinoma; ATA, American Thyroid Association; MSKCC-NY, Memorial Sloan Kettering Cancer Center-New York. *P<0.05.

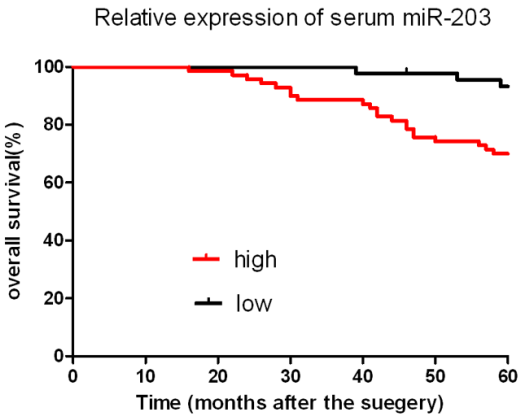

Figure 3. The overall survival and serum expressions of miR-203 by analysis of Kaplan-Meier survival curves. The overall survival rates in patients with high expressions of miR-203 was much lower than those with low expressions of miR-203 (P<0.001).

0.755, with 95% confidence interval of 0.651 to 0.860 (P<0.001), which was shown in **Figure 2**. The cut-off value of miR-203 was 3.560, with a sensitivity of 71.1% and a specificity of 80.0% respectively.

Clinical and pathological characteristics and recurrence of PTC

PTC patients enrolled in this study were divided into two groups, recurrent group and non-recurrent group. The summary of the clinical and pathological characteristics of patients in two groups was comprehensively presented in **Table 1**. We observed that patients who had PTC recurrence were with a higher ATA risk, higher MSKCC-NY risk, a bigger tumor size, higher TNM stages, increased serum miR-203 levels, and higher rates of cervical lymph node metastasis, vascular invasion, perineural invasion and extra thyroidal extension (P<0.05). Then we used univariate and multivariate Cox proportional hazards analysis to evaluate the association of clinicopathological characteristics and serum miR-203 expressions with recurrence in order to explore the potential prognostic biomarkers for PTC recurrence. The results of **Table 2** have shown that the TNM stage (HR=4.54, 95% CI: 1.10-9.98, P=0.019) and serum levels of miR-203 (HR=1.43, 95% CI: 1.15-1.85, P=0.014) were both significantly associated with the recurrence of PTC.

Clinicopathological characteristics and overall survival rate

As presented in **Figure 3**, high expressions of miR-203 were significantly associated with lower overall survival rate of PTC patients by the Kaplan-Meier analysis with log-rank test. The correlations between clinicopathological characteristics and overall survival rate in

Table 3. Multivariate analysis of overall survival in 165 patients with PTC by Cox regression analysis

	Overall survival		
Parameter	HR	95% CI	*P* value
Age	1.24	0.36-2.93	0.40
Gender	1.01	0.43-2.81	0.51
ATA risk	1.32	0.35-3.14	0.31
MSKCC-NY risk	3.19	0.76-7.22	0.14
Tumor size	2.38	0.76-4.35	0.22
Cervical lymph node metastasis	3.14	2.24-6.89	0.017*
TNM stage	2.64	1.67-6.78	0.010*
Vascular invasion	1.24	0.45-4.31	0.71
Perineural invasion	4.98	1.65-10.53	0.023*
Extrathyroidal extension	0.91	0.29-3.65	0.79
Histological subtype	1.09	0.24-6.53	0.61
Serum miRNA-203 expression	6.75	1.43-15.43	0.001*

PTC, Papillary thyroid carcinoma; ATA, American Thyroid Association; MSKCC-NY, Memorial Sloan Kettering Cancer Center-New York; HR, Hazard ratio; CI, Confidence interval. *P<0.05.

patients with PTC by utilizing multivariate analysis with Cox regression analyses were presented in **Table 3**. The results suggested that serum miRNA-203 expression (HR=6.75, 95% CI: 1.43-15.43, P=0.001) was an independent prognosis factor for the overall survival rate of PTC patients as well as cervical lymph node metastasis, TNM stage and perineural invasion.

Discussion

This study aimed at the identification of potential prognostic biomarkers for predicting recurrence in PTC patients, which would be beneficial for identifying extensive surgeries or the need for prophylactic lymph node resections. However, no effective and non-invasive biomarkers for recurrence and prognosis of PTC have been found to date and great efforts are carried out in this pursuit [1]. Herein, we evaluate the clinical and pathological characteristics in PTC patients who had undergone total thyroidectomy at our hospital in this study. Of all the enrolled 165 PTC patients, 48 cases have developed tumor recurrence within 60 months, with a recurrence rate of 29.1%. As shown in **Table 1**, patients who had PTC recurrence were with a higher ATA risk, higher MSKCC-NY risk, a bigger tumor size, higher TNM stages, increased serum miR-203 levels, and higher rates of cervical lymph node metastasis, vascular invasion, perineural invasion and extra thyroidal extension (P<0.05). The results of univariate and multivariate Cox proportional hazards analysis suggested that TNM stage and serum miR-203 levels were independent prognostic factors for PTC recurrence. Previous studies have revealed that larger tumors were associated with increased incidence of nodal spread and worse disease-free survival [17, 18]. Tumor size was also proved to be a risk factor for central lymph node metastases in PTC patients [19, 20]. Other parameters (ATA risk, MSKCC-NY risk, cervical lymph node metastasis, etc.) were not important predictive factors for PTC recurrence, which was in accordance with other studies [21, 22]. We also examined the serum levels of miR-203 in recurred and non-recurred PTC patients by qRT-PCR. Our analysis demonstrated that serum miR-203 expressions were significantly increased in PTC patients with tumor recurrence in comparison with those with no recurrence, which was in accordance with results from other studies [14]. To validate the predicative power of serum miR-203 for PTC recurrence, we conducted ROC analysis which suggested that serum miR-203 expression was a significant predicative factor for PTC recurrence with an AUC of 0.755, a sensitivity of 71.1% and a specificity of 80.0% respectively. The epithelial-mesenchymal transition (EMT) is a key process in cancer metastasis and it can convert polarised immotile epithelial cells into motile, invasive mesenchymal cells and enable cancer cells to gain stem cell characteristics and an aggressive malignant phenotype [23, 24]. MiR-203 has been reported to directly suppress EMT activators including SNAI1/2 and zinc finger E-box binding homeobox 2 (ZEB2) [25, 26]. MiR-203 has been proved to be a putative tumor suppressor gene and a target of promoter hypermethylation [27]. MiR-203 has also been reported to inhibit cell invasion, migration, proliferation and tumor angiogenesis in many types of tumor cells [28, 29]. Accumulating data in recent years have also convincingly demonstrated that high levels of serum miR-203 were a promising non-invasive prognostic and metastasis-predictive biomarker in patients with colorectal cancer [30]. The association between miR-203 and PTC recurrence were not found in the literature data

and this present study firstly identified miR-203 as a potential recurrence predictor for PTC.

Moreover, we also investigated the association between serum miR-203 levels with 5-year overall survival rate of PTC patients. Results from Kaplan-Meier survival curves showed that high serum miR-203 serum expression was significantly associated with poor overall survival in PTC patients (see **Figure 3**; log-rank P<0.001). The multivariate analysis with Cox regression analysis demonstrated that serum miR-203 expression was an independent prognosis factor for the overall survival rate of PTC patients as well as other clinicopathological characteristics including cervical lymph node metastasis, TNM stage and perineural invasion. We propose thatserum miR-203 expression is a promising biomarker for the identification of PTC with recurrence who need adequate intervention (such as administration of chemotherapy, surgical resection, etc.) to gain a longer overall survival rate.

In conclusion, this study demonstrated the potential role of serum miR-203 expression as a non-invasive predicative biomarker for the recurrence and prognosis of PTC.

Disclosure of conflict of interest

None.

Authors' contribution

JR Z participated in the conception and design, data collection, statistical analysis and wrote the manuscript. JJ L participated in the conception and design and data collection.

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