



miR-21 facilitates the diagnostic value of miR-138 for papillary thyroid cancer in formalin-fixed paraffin-embedded tissues

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Background: miR-138 was thought to be anti-tumor miRNA for its ability of suppressing tumor growth. The objective of this study was to evaluate the potential predictive value of miR-138 and its combination with miR-21 in formalin-fixed paraffin-embedded (FFPE) tissues of papillary thyroid cancers (PTCs).

Methods: miR-138/miR-21 expression was tested in 101 PTC and 51 benign thyroid nodule (control) patients using quantitative real-time PCR. Clinical and pathological characteristics of enrolled subjects were obtained from medical records.

Results: miR-138 was significantly down-regulated in PTC compared with controls (median 0.0122 vs. 0.0943), and miR-138 exhibited capability of discriminating PTC from controls, with a diagnostic accuracy of 71.1% (sensitivity 84.3%, specificity 49.5%). A combination of miR-138 and miR-21 increased the diagnostic accuracy to 79.1% (sensitivity 73.3%, specificity 76.5%). Multivariate logistic regression confirmed the association between miR-138/miR-21 expression and PTC diagnosis. Moreover, miR-138 expression was further down-regulated in PTC with capsule invasion compared with PTC tissues without capsule invasion (median 0.0043 vs. 0.0373, $P=0.003$).

Conclusions: MiR-138 expression was not only associated with onset of PTC, but also the aggressiveness of PTC. A combination of miR-138 and miR-21 enhanced the diagnostic value of individual biomarkers.

Keywords: Biomarkers; papillary thyroid cancer (PTC); formalin-fixed paraffin-embedded tissue; miR-138; miR-21

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Introduction

Thyroid cancer is the most common endocrinology cancer. In the United States, the incidence of new thyroid cancer cases was 13.9 per 100,000 men and women per year, based on the data 2009–2013, accounting for 3.8% of new cancer cases (1). Among all the thyroid cancers, papillary thyroid

cancer (PTC) is most common (1). In the primary test for thyroid cancer—fine needle aspiration biopsy (FNAB)—up to one third of the biopsy results were indeterminate, without clear indication for diagnosis (2). In this case, biomarkers to differentiate malignant and benign lesions are of our interest.

MicroRNAs are small non-coding RNAs typically with 18–21 nucleotides (3,4). MicroRNAs work in post-transcriptional gene silencing, thus triggering either translational repression or RNA degradation (5). In this way, miRNAs play important roles in many regulatory pathways including tumor growth (6) and invasion (7).

Normally fresh and frozen tissues are considered as proper samples for RNA research. However, given the time and money cost of prospectively fresh/frozen tissues collecting, formalin-fixed paraffin-embedded (FFPE) tissues were considered as potential alternative resource for biomedical research (8). Because of the shorter length of miRNAs, they seemed to be more stable and can be detected in FFPE tissues, when RNA appeared to degrade in the same samples (9). Meanwhile, miRNA expression in FFPE tissues was highly correlated with miRNA expression in matched fresh/frozen tissues (8). Therefore, given the comparative easy availability and low cost of obtaining FFPE tissues, they might be an ideal source for miRNA analysis.

miR-138 and miR-21 play vital roles in tumor biology. miR-138 was shown to suppress tumor growth (10–12), reverse drug resistance (13,14) in cancers, and inhibit differentiation in stem cells (15,16). In addition, miR-138 was also associated with epithelial-mesenchymal transition (16–18), which plays important role in cancer development and progression. On the contrary, miR-21 was suggested to promote cancer (19,20). Recently, You *et al.* (21) have reported significant differences in expression levels of miR-21 and miR-138 when comparing colorectal cancer and normal adjacent tissues. Results showed that the expression level of miR-21 in colon cancer tissues was significantly higher comparing to adjacent healthy tissues, while miR-138 was lower in colon cancer tissues. And they indicated that miR-21 and miR-138 may be involved in the regulation of colon cancer cell proliferation. A study on the mechanism of prostate cancer metastasis showed that α -solanine reversed EMT by down-regulating the expression of miR-21 and up-regulating the expression of miR-138, which may be involved in the anti-metastatic mechanisms of α -solanine in human prostate cancer cell (22).

In thyroid cancers, miR-21 has been proved to be an oncogenic factor (23,24), while miR-138 was much less studied. Several studies have reported overexpression of miR-21 in PTCs, as compared with levels in non-cancer thyroid tissues (23–25), and miR-21 was associated with the risk of recurrence of PTCs (26). MiR-21 was also reported to be significantly overexpressed in medullary

thyroid cancer (MTC) by comparison with adjacent normal thyroid tissues (27). In addition, Chu *et al.* (28) indicated that increased expression of miR-21 was related to the worse prognosis with persistent and metastatic disease in MTC patients. More recently, Samsonov *et al.* (29) have identified that miR-21 was overexpressed in the plasma exosomes of patients with follicular thyroid cancer (FTC), than those in PTC patients, while miR-181a was higher expressed in PTC patients than in patients with FTC. And comparative assessment of miR-21-5p/miR-181a amplification ratios contributed to distinguish patients with FTC from patients with PTC with 100% sensitivity and relatively high specificity. In several studies, miR-138 was shown to be down-regulated in thyroid cancers (30–32). Of note, significantly reduced expression of miR-138 was observed in Hürthle cell thyroid cancer (HCTC), as well as patients with distant metastases, compared to normal thyroid tissues (33). While in very limited study, miR-138 was shown to be up-regulated in malignant thyroid cancers (34). To our knowledge, no reasonable explanation was given for that inconsistency. Furthermore, a racial disparity in miR-138 expression was demonstrated by Suresh *et al.* (30). They found that miR-138 distributed differently in Caucasian Americans and African Americans between malignant and benign thyroid lesions. This study gave us a hint, that the possibility of different miR-138 distribution pattern might exist in Chinese population. Up to now, most of the studies on miR-138 were carried out *in vitro* studies, e.g., cell lines (32), or in other populations (30–32,34) but not in Chinese population.

On the other hand, since miR-138 and miR-21 play countered roles in cancer development and progression, and both are involved in common cancer regulatory net, for example, related to epithelial-mesenchymal transition (18,35–37), we extrapolate that the combination of both miRNAs might work in different ways compared with individual miRNAs.

Therefore, the objectives of the present study were (I) to investigate the diagnostic role of miR-138 in Chinese PTC patients; (II) to explore the possible interaction between miR-138 and miR-21 in PTC patients.

Methods

Patients and samples

FFPE tissue samples from 101 PTC and 51 thyroid benign nodules (control) patients. FFPE samples were

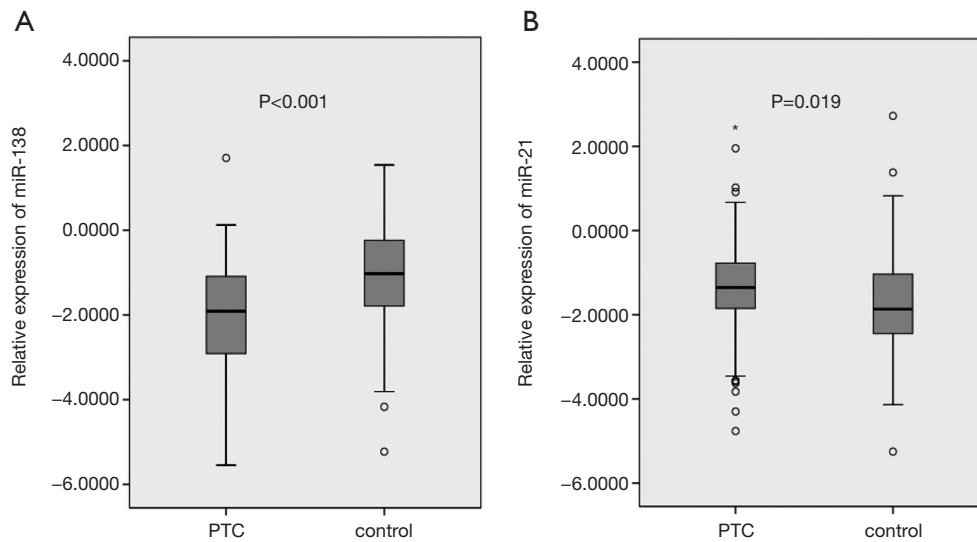


Figure 1 Relative miR-138 (A) and miR-21 (B) expression in 101 papillary thyroid cancers and 51 controls. miRNA expressions were presented after log₁₀ transformation. Thick line in the box represented the median; upper- and lower-sides of box represented the 75% and 25% percentile; circles represented mild outliers; stars represented extreme outliers, error bars represented the maximum and minimum of “normal” distribution.

retrospectively collected from Affiliated People’s Hospital of Jiangsu University. All samples were obtained based on the guidelines of hospital’s protocol including patient consent and specimen collection. This study was approved by the Institutional Review Boards of Affiliated People’s Hospital of Jiangsu University (No. 20150089). The diagnosis of thyroid cancers was based on pathological and clinical features of cancers.

RNA extraction and reverse transcription

Total RNA was extracted in all the samples with the RecoverALL™ Total Nucleic Acid Isolation Kit (Ambion, AM1975). Reverse transcription was made with miScript Reverse Transcription Kit (Qiagen, 218061). All the procedures were performed according to the instructions of manufacturers.

Quantitative real-time PCR

Quantitative real-time PCR was performed with miScript SYBR green PCR Kit (Qiagen, 218073), according to the protocol provided by the manufacturer. The primer in the Kit was used as universal forward primer. The sequences of reverse primers were: miR-138, 5'-AGCTGGTGTGTTGTAATCAGGCCG-3'; miR-21,

5'-GAGCTTATCAGACTGATGTTGA-3'. All the miRNA expression was normalized by U6 expression. The condition of PCR was described in details before (38).

Statistical methods

All the statistical analyses were performed with SPSS 20.0. miR-138/miR-21 expression was presented as median of copies normalized to U6 expression. Receiver operator characteristic (ROC) curves were drawn to show the diagnostic value of miRNA(s) for PTC. Univariate and multivariate logistic regressions were performed to analyze the association between miRNA expression and PTC. MiRNA expression in cancer patients by clinical and pathological characteristics was presented as quartile, and Mann-Whitney U test were applied to compare miRNA expression between groups.

Results

miR-138/miR-21 expression in thyroid cancers and controls

miR-138 exhibited significantly lower, and miR-21 showed significantly higher expression in PTC patients compared with controls (both $P < 0.05$) (Figure 1A,B; Table 1). The

Table 1 Expression of miR-138/miR-21 and their diagnostic value for papillary thyroid cancer

miRNA	Median (cancer)	Median (control)	P	AUC	95% CI	Cutoffs	Sensitivity (%)	Specificity (%)
miR-138	0.0122	0.0943	<0.001	0.711	0.624–0.798	0.0099	84.3	49.5
miR-21	0.0442	0.0136	0.019	0.617	0.520–0.713	0.0138	76.2	51.0
miR-21/miR-138	4.6447	0.1544	<0.001	0.791	0.713–0.868	1.0124	73.3	76.5

The AUC of miR-138 was for controls. AUC, areas under the curve; CI, confidence interval

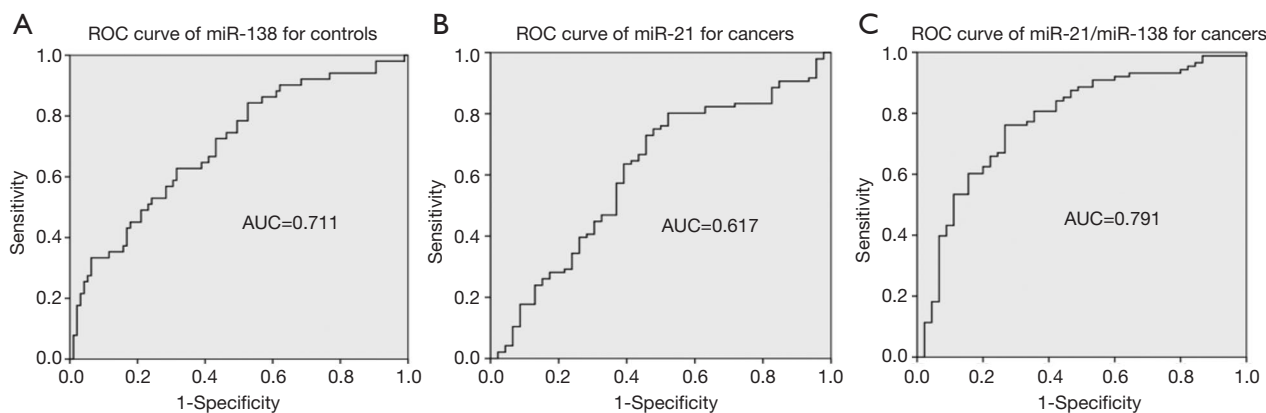


Figure 2 ROC curve of miR-138, miR-21 and the ratio of miR-21 and miR-138 expression. The area under the curve (AUC) indicated the accuracy for discriminating papillary thyroid cancer and controls in sensitivity and specificity. The combination of miR-138 and miR-21 produced a larger AUC than the individual miRNAs.

median of miR-138 expression in PTC patients was almost eight folds lower than that in controls (0.0122 *vs.* 0.0943), while the median of miR-21 expression in PTC patients was 3.3 times higher than that in controls (0.0442 *vs.* 0.0136) (*Table 1*).

Diagnostic value of miRNA-138/miR-21 for PTC

ROC curve showed the capability of miRNA-138/miR-21 to discriminate PTC and controls. As indicated in *Table 1* and *Figure 2*, both miR-138 and miR-21 had a significant discriminating capability between PTCs and controls (71.1% and 61.7%, respectively, both $P < 0.05$). As shown in *Table 1*, with the optimized cut-offs, both miR-138 and miR-21 produced a relatively high sensitivity (84.3% and 76.2%, respectively), but only moderate specificity (49.5% and 51.0%, respectively). However, a combination of both produced a higher diagnostic accuracy (79.1%), as well as an increased specificity (76.5%) and slightly decreased sensitivity (73.3%) (*Table 1*). In addition, multivariate logistic regression showed that miR-138 had a protective role (OR = 0.456, 95% CI: 0.326–0.639) and miR-21 had

a predictive value (OR = 1.874, 95% CI: 1.272–2.761) for PTC, despite that the univariate regression result for miR-21 was not significant ($P = 0.087$) (*Table 2*).

miR-138/miR-21 expression by clinical and pathological characteristics in PTC patients

To further investigate whether miRNA expression was associated with aggressive features of PTC, we compared the miR-138 and miR-21 expression in cancer patients by clinical and pathological features. As shown in *Table 3*, miR-138 expression was further down-regulated in PTC with aggressive features, including advanced tumor stage (stage III, IV), capsule invasion, lymph node metastasis and extrathyroidal extension, only with statistical significance in PTC with capsule invasion ($P = 0.003$). miR-21 expression did not differ by clinical and pathological characteristics.

Discussion

In the present study, we found that miR-138 expression in PTC was significantly down-regulated, while miR-21 was

Table 2 Univariate and multivariate logistic regression of miR-138/miR-21 for papillary thyroid cancer

Univariate/multivariate	β	S.E.	Wald	P	OR	95% CI
Univariate						
miR-138	-0.590	0.151	15.249	<0.001	0.554	0.412–0.745
miR-21	0.246	0.143	2.936	0.087	1.279	0.965–1.694
Multivariate						
miR-138	-0.785	0.172	20.887	<0.001	0.456	0.326–0.639
miR-21	0.628	0.198	10.098	0.001	1.874	1.272–2.761

miRNA expression was log₁₀ transformed in the logistic regression model. CI, confidence interval.

significantly up-regulated compared with benign thyroid nodules (both $P < 0.05$) (Table 1, Figure 1). Both miR-138 and miR-21 produced a significant diagnostic accuracy (71.1% vs. 61.7%, respectively), and a combination of them produced a higher diagnostic accuracy (79.1%) (Table 1, Figure 2). The logistic regression of miRNA expression for PTC confirmed the diagnostic value of the combination of both miRNAs, showing as the protective role of miR-138 against (OR = 0.456, 95% CI: 0.326–0.639, $P < 0.001$) and predictive value of miR-21 for PTC (OR = 1.874, 95% CI: 1.272–2.761, $P = 0.001$) (Table 2). Further comparison of miRNA expression in PTC patients by clinical and pathological characteristics showed that miR-138 expression was significantly lower in cancers with capsule invasion (Table 3), suggesting that miR-138 was associated with aggressiveness of PTC.

First we analyzed the miR-138 and miR-21 expression in PTC and benign thyroid nodule tissues. The up-regulation of miR-21 expression in PTC in our data was consistent with previous studies (23,25). Regarding the much less studied miR-138, in most cases, miR-138 was shown to be down-regulated in PTC patients (30–32), though limited study showed a up-regulation of miR-138 in thyroid cancers of follicular origin (34), and a racial disparity was also reported by Suresh *et al.* (30). Suresh's data agreed on the general down-regulation of miR-138 in PTC ($P = 0.0001$), but to a larger magnitude in African Americans than in Caucasian Americans. Though with limitation of small sample size and lack of statistical significance, this study showed that ethnicity might be another confounding factor for miR-138 expression. In the present study, we found that miR-138 expression in Chinese PTC patients was eight times lower than that in benign thyroid nodules ($P < 0.001$, Table 1), which was consistent with Cong's study in another Chinese population (39). Our data supported the general

opinion of down-regulated miR-138 in PTCs, and provided new evidences in Chinese population, corresponding to the racial disparity in miR-138 distribution. The diagnostic accuracy of miR-138 for PTC differed in studies, ranging from some 70% to 90% (34,39). In our data, the diagnostic accuracy was 71.1%. It is worth noting that, for the first time, to our knowledge, we found that a combination of miR-138 and miR-21 could increase the diagnostic accuracy (in our case from 71.1% to 79.1%); meanwhile, sensitivity and specificity were balanced to a more appropriate status for a diagnostic tool (sensitivity 73.3%, specificity 76.5%) (Table 1). Compared with univariate logistic regression, multivariate logistic regression revealed an intensified protective role of miR-138 (multivariate vs. univariate OR = 0.456 vs. 0.554) against and a stronger predictive role of miR-21 (multivariate vs. univariate OR = 1.874 vs. 1.279) for PTC (Table 2). Interestingly, the predictive role of miR-21 has turned to be statistically significant after controlling for miR-138 (P value turned from 0.087 to 0.001) (Table 2). These results also supported the increased diagnostic accuracy of the combination. Furthermore, plasma exosomal miR-21 expression also helped to differentiate follicular from PTCs, demonstrated as the significantly higher miR-21 expression in FTCs (29).

The increased diagnostic accuracy of combined biomarkers (Table 1) and stronger association between combined biomarkers and PTC (Table 2) suggested a possible practical diagnostic tool in clinical setting. Since the high correlation of miRNA expression between FFPE samples and fresh/frozen samples, this diagnostic tool would help to differentiate PTC from benign nodules, particularly for those patients who had indeterminate FNAB results, thus decreasing unnecessary thyroid operations (40).

Furthermore, the down-regulated miR-138 expression in cancers with capsule invasion (Table 3) suggested that miR-

Table 3 miR-138/miR-21 expression by clinical and pathological characteristics in papillary thyroid cancer

Patients' characteristics	N	miR-138				miR-21			
		Median	Q1	Q3	P	Median	Q1	Q3	P
Age (years)					0.230				0.558
<45	46	0.0053	0.0011	0.0429		0.0417	0.0072	0.1546	
≥45	55	0.0160	0.0012	0.1104		0.0515	0.0190	0.1703	
Gender					0.319				0.838
Male	20	0.0262	0.0018	0.1548		0.057	0.0107	0.1134	
Female	81	0.0099	0.0011	0.0860		0.0431	0.0140	0.1739	
Recurrence					0.063				0.843
No	98	0.0098	0.0011	0.0739		0.0449	0.0156	0.169	
Yes	3	0.9072	0.0680	–		0.012	0.0078	–	
T category					0.818				0.189
1 and 2	83	0.0099	0.0011	0.0715		0.0409	0.0141	0.1154	
3 and 4	18	0.0132	0.0012	0.1445		0.1159	0.0110	0.8737	
N category					0.650				0.712
N0	39	0.0169	0.0011	0.0909		0.0401	0.0096	0.3879	
N1a,b	62	0.0087	0.0012	0.0836		0.0557	0.0163	0.1556	
Metastasis					–				–
No	101	0.0122	0.0012	0.0860		0.0442	0.0140	0.1694	
Yes	0	–	–	–		–	–	–	
Tumor stage					0.815				0.704
I–II	69	0.0130	0.0012	0.0764		0.0402	0.0092	0.2861	
III–IV	32	0.0087	0.0009	0.1047		0.0602	0.0218	0.144	
Multifocality					0.523				0.163
No	77	0.0090	0.0012	0.0704		0.0600	0.0176	0.1952	
Yes	24	0.0249	0.0010	0.1712		0.0323	0.0083	0.1190	
Capsule invasion					0.003**				0.615
No	51	0.0373	0.0018	0.2775		0.0515	0.0139	0.1703	
Yes	50	0.0043	0.0007	0.0310		0.0401	0.0128	0.1529	
Lymph node metastasis					0.780				0.660
No	39	0.0169	0.0011	0.0909		0.0401	0.0096	0.3879	
Yes	62	0.0087	0.0013	0.0836		0.0557	0.0163	0.1556	
Extrathyroidal extension					0.766				0.584
No	96	0.0122	0.0012	0.0908		0.0449	0.0162	0.1699	
Yes	5	0.0022	0.0011	0.0746		0.0139	0.0051	0.3964	

**; P<0.01.

138 dysregulation was not only associated with the onset of PTC, but also the aggressiveness of PTC. Similar results were also observed in Americans (31) and another Chinese population (39). In Yip's study, miR-138 expression in aggressive PTC was only half of nonaggressive PTC, but no statistical significance was seen ($P=0.189$) (31). In Cong's study, miR-138-1 expression was significantly lower in PTC with extrathyroidal extension ($P=0.005$) and in PTC patients with advanced tumor stage (III/IV stage, $P=0.045$) (39). In our data, we did not find significant difference in miR-138 expression in PTC patients stratified by Tumor stage, lymph node metastasis or extrathyroidal extension, which are common indicators for aggressiveness of PTC. However, miR-138 expression in PTC samples with these "aggressive" features were lower than those without these features, in spite of lack of statistical difference. The lack of significance might be caused by the characteristics of enrolled patients and limited sample size. In our study, for most cases, PTC was diagnosed in routine test and exams, and patients enrolled were generally mild cases. The relative small number of "aggressive" tumors may hide the difference of miR-138 expression. However, the significant difference in miR-138 expression between patients with/without capsule invasion has supported the miR-138 role in the aggressiveness of cancers.

On the other hand, *in vitro* study, miR-138 expression in anaplastic thyroid cancer, a more aggressive type of thyroid cancer, was also down-regulated compared with PTC, a relative mild type of thyroid cancer (32). This finding supported the aggressive feature of miR-138 down-regulated thyroid cancers, though from comparison between pathological types.

Conclusions

In conclusion, we revealed that miR-138 down-regulation was not only associated with onset of PTC, but also the aggressiveness of PTC. And for the first time, we showed that a combination of miR-138 and miR-21 could increase the diagnostic accuracy for PTC. A combination of miR-138 and miR-21 could be used as a diagnostic tool to differentiate PTC from benign lesions. Further studies will be carried out to disclose the mechanism of miRNA-138 to regulate tumor genesis and progression, and the interaction with miR-21.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.08.14>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This retrospective study was approved by the ethics committee of the Affiliated People's Hospital of Jiangsu University (No. 20150089). All samples were obtained based on the guidelines of hospital's protocol including patient consent and specimen collection.

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