Combination of serum microRNAs and ultrasound profile as predictive biomarkers of diagnosis and prognosis for papillary thyroid microcarcinoma

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Abstract. Circulating microRNAs (miRNAs) are promising diagnostic markers in various types of cancers, including papillary thyroid carcinoma (PTC). However, there is sparse information reported with regards to miRNA expression in papillary thyroid microcarcinoma (PTMC) or concerning the role of a combination of miRNAs and ultrasound (US) in the diagnosis of PTMC before surgery. Therefore, we designed a study that aimed to evaluate miRNA expression levels and their potential associations with US findings and determine whether miRNAs could be used as diagnostic and prognostic biomarkers of PTMC. miR-222, miR-221, miR-146b and miR-21 levels were determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in serum from 58 patients with PTMC and 47 with PTC, 35 patients with benign thyroid nodules (BTN) and 40 control subjects. Expression levels of the four miRNAs in serum were evaluated before and after surgery. The results indicated that miR-222, miR-221, miR-146b and miR-21 expression levels were higher in the PTMC samples than in those from the BTN and control groups and the combination of miRNAs and US had a high sensitivity and specificity for discrimination between BTN and PTMC by receiver operating characteristic (ROC) curve analysis and improved the accuracy of diagnosis of PTMC before surgery. In addition, serum miRNA expression levels were significantly related to poor prognostic factors including metastatic lymph nodules (MLNs), multifocal and bilateral lesions, advanced stage and high-risk PTMC patients. The miRNA expression levels in serum from PTMC patients were rapidly reduced after surgery compared with levels before surgery. In addition, we also analyzed the miRNA expression levels in serum from patients who were divided into two groups according to factors indicating a good or poor prognosis associated with PTMC after surgery. The results suggested that after surgery, the miR-222, miR-146b and miR-21 expression levels were significantly higher in the poor prognosis group compared with these levels in the good prognosis group. Serum miRNA expression levels helped distinguish between benign and malignant nodules and were associated with a poor prognosis in PTMC. Circulating miRNAs may be useful as follow-up biomarkers and as diagnostic and prognostic tools.

Introduction

Papillary thyroid microcarcinoma (PTMC) is a subtype of papillary thyroid carcinoma (PTC), which is defined by the WHO as measuring 1.0 cm or less in the greatest dimension of the tumor (1). There is a well-documented, worldwide trend showing an rapid increasing incidence of PTC in recent decades (2-4). This increase has been predominantly an increase in PTMC, accounting for 50% of PTCs (5-7).

High-resolution ultrasound (US) is a widespread technique used as a first-line diagnostic tools for PTMC before surgery. Although certain US findings are suggestive of malignancy, their predictive value is inconsistent, and the accumulated predictive value of US characteristics and basic clinical features range between 30 and 48% (8). A recent study has reported that 17.2-22.6% of nodules were finally confirmed as benign despite the presence of suspicious US features (9,10). US-guided fine-needle aspiration biopsy (US-FNAB) is currently the most important method for determining a diagnosis of PTMC and could serve as a supplemental method for non-diagnostic and indeterminate nodules on US. However, the rate of non-diagnostic and indeterminate FNA remains high at around 30%. Thus, these patients undergo surgery, but fewer

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than 20% of surgically removed nodules are malignant (11,12). Therefore, one of the key issues for PTMC patients is detecting new indices and improving the accuracy of diagnosis.

Although the prognosis of PTMC is good, there is still a risk of recurrence and metastasis, including distant metastasis. Currently, it has been recognized that recurrences are related to metastatic lymph nodules (MLNs), extrathyroidal extension (ETE) and multifocal lesions; yet, there is still no accurate method to distinguish PTMC patients with a poor prognosis and risk of recurrence from those with an excellent prognosis. Therefore, PTMC is not a uniform category. Generally, for future clinical work, it is crucial to determine the clinical and especially the molecular parameters that define a small group of PTMCs with an aggressive biological behavior.

miRNAs are small noncoding RNAs that negatively regulate gene expression either through inhibition of mRNA translation or by promoting mRNA degradation. There is also evidence that miRNAs are involved in central biological processes in cancers, including development, organogenesis, tissue differentiation, the cell cycle and metabolism (13-15). Several miRNAs (miR-222, miR-221, miR-146b and miR-21) have been reported to be consistently deregulated in PTC and have been demonstrated to distinguish benign from malignant nodules and have a reasonable diagnostic accuracy in cell lines, FNA and tumor tissue specimens (16-19) To date, there are no reports that have addressed the possibility of evaluating circulating miR-222, miR-221, miR-146b and miR-21 levels in patients with PTMC. Therefore, the objective of this study was to analyze serum miRNA expression before and after surgery in PTMC and to determine their relationship with clinicopathologic factors to elucidate whether the abnormal expression of specific miRNAs correlates with the diagnosis and prognosis of PTMC. In addition, we aimed to elucidate whether there were differences in the expression of miRNAs between PTC and PTMC groups. We also evaluated the preoperative US of these patients with PTMC and compared miRNAs with US with regard to their sensitivity and specificity of the diagnosis of PTMC before surgery. In addition, we also analyzed the combination of miRNAs and US for their diagnostic value for PTMC before surgery and elucidated whether their use could improve the accuracy of diagnosis.

Materials and methods

This study was granted ethical approval by the Institutional Review Board of Harbin Medical University. Written informed consent was obtained from all participants involved in the study. The reference code for the ethical approval was no. HMUIRB20150020.

Patients. A total of 58 patients with PTMC, 47 patients with PTC with diameters >1 cm, 35 patients with BNT and 40 controls were enrolled. All patients were recruited at the Department of Thyroid Surgery of the Second Affiliated Hospital of Harbin University (Heilongjiang, China) between September 2013 and December 2016. Additional inclusion criteria were patients with BTN who had single or multiple nodules, patients with PTMC and patients with PTC who were undergoing a thyroidectomy and a final diagnosis was based on the pathological results by two experienced pathologists at the Department of Pathology and at the Department of Thyroid Surgery of the Second Affiliated Hospital of Harbin University. In addition, 40 control subjects were enrolled from the Physical Examination Center of the Second Affiliated Hospital of Harbin University (Heilongjiang, China) between September 2013 and December 2016. The control subjects were age- and sex-matched volunteers without a current or previous history of any other types of cancer and were confirmed to not have thyroid disease based on a neck US and thyroid hormone measurements.

Preoperative US of patients and controls. All of the controls and patients, including those with PTMC, PTC and BTN had undergone preoperative thyroid US, followed by thyroidectomy and histopathology examination. US procedures were performed prospectively by two ultrasonic physicians with 3-5 years of experience and specialization in thyroid US. Thyroid US was performed with an 8-15 MHz linear-array transducer (HI VISION 500, Japan). The following features of the lesions, using criteria obtained from published reports (20-22), were assessed: Size of the tumor, lesion number, marked hypoechogenicity location, extrathyroidal extension (ETE), lesion echogenicity, cystic degeneration microcalcification, well-defined or ill-defined boundary and a halo sign, The results of US were scored by achieved agreement between two radiologists.

We calculated the probability of malignant nodules using two malignant risk systems including web-based TIRADS (www.gap.kr/thyroidnodule.php) and K-TIRADS, and American Thyroid Association (ATA) guidelines (23,24). Suspicious lesions mainly included those with findings of a score of ≥8 with the web-based TIRADS, high suspicion nodules with K-TIRADS, and high suspicion with the ATA guidelines (23,24).

Samples and RNA extraction. Venous blood samples were collected in vacuum-sealed blood collection tubes from all control subjects and from patients with PTMC and BTN on the day of surgery. The first 1 ml of samples was discarded to reduced possible contamination with miRNA from the dermal plug from the venous puncture. Within 1 h of collection, the samples were centrifuged at 1,900 x g for 15 min at 4˚C. The supernatant was carefully transferred into an RNase-free tube and centrifuged at 12,000 x g for 10 min at 4˚C to remove additional cellular debris and to minimize the contamination of the cell-free nucleic acid by DNA and RNA derived from damaged blood cells. The serum samples were stored at -80˚C until RNA extraction.

Total RNA was isolated from 200 μl of serum according to the manufacturer's protocol using a miRNeasy serum/plasma kit (Qiagen GmbH, Hilden, Germany). The concentrations of all RNA samples were quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Wilmington, DE, USA). The concentrations of RNA extracted from serum ranged between 14.3 and 26.8 ng/ml.

Selection of miRNAs. The miRNA candidates to be tested were selected based on the following process. Firstly, miRNAs that were enriched in normal thyroid tissues and significantly unregulated in PTC compared with normal thyroid tissues and BTN were selected (25-29). Secondly, we selected miRNAs reported as being upregulated in tumor tissue to determine whether their expression was also elevated in serum derived from patients with PTMC. Finally, miRNAs that were significantly associated with

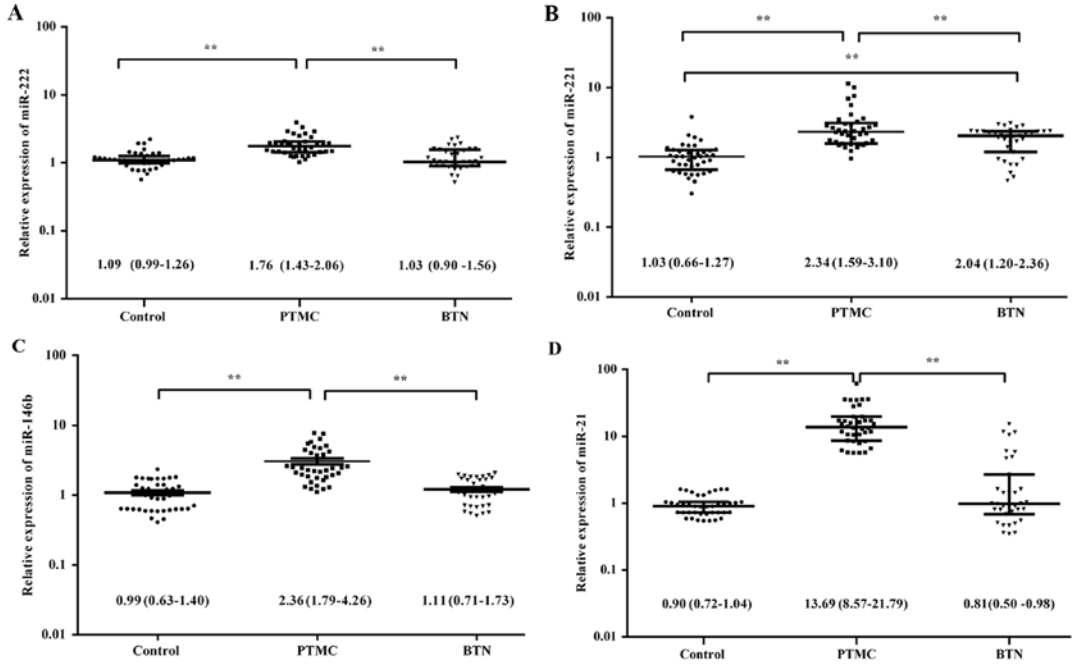

Figure 1. Diagrams of the Mann-Whitney U test results, showing that the relative expression levels of miR-222 (A), miR-221 (B), miR-146b (C) and miR-21 (D) in the PTMC group (n=58) were significantly higher than level in the control (n=40, $^{**}P<0.01$) and BTN (n=35, $^{**}P<0.01$) groups. The relative expression of miRNAs is presented as the median and interquartile range $2^{-\Delta\Delta Ct}$. PTMC, papillary thyroid microcarcinoma; BTN, benign thyroid nodules.

a poor prognosis of PTC were selected (25-32). Based on this selection procedure, 4 miRNAs (miR-222, miR-221, miR-146b and miR-21) were selected as candidate targets for the serum miRNA assay, and we evaluated whether these miRNAs were associated with the diagnosis and prognosis of PTMC.

qRT-PCR analysis of miRNAs. First-strand cDNA synthesis of miRNA was performed using a miRcute miRNA First-Strand cDNA synthesis kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. Reverse transcription was conducted on a GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Briefly, 5 μl of total RNA was extracted from plasma, polyadenylated by poly(A) polymerase and reverse transcribed into cDNA according to the manufacturer's protocol. RT-qPCR was performed in duplicate using the SYBR Green PCR Master Mix (miRcute miRNA qPCR detection kit; Tiangen Biotech Co., Ltd.) with the Stratagene Mx3000PTM real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). miRNA-specific primer sequences were designed based on the miRBase database by Primer Premier 5.0 and are presented in Table I.

Some studies have reported that miR-16 has been used as an endogenous reference for serum and plasma miRNA examination, thus miR-16 was selected as an endogenous control in this study (33-35). The relative expression was obtained using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$). The fold-change in the expression of each miRNA was determined by comparing the mean $2^{-\Delta\Delta Ct}$ values of the PTC and BTN groups to the mean $2^{-\Delta\Delta Ct}$ value of the control group (36).

Statistical analysis. Statistical analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). The Mann-Whitney U test, Kruskal-Wallis H test and Scheffe's Post Hoc Multiple Comparisons test were used to determine the significance of the different levels of miRNA expression. Fisher's exact test was used to measure the characteristics in the PTMC and PTC groups. ROC curves were used to analyze the diagnostic utility of differentially expressed miRNAs. The optimal cut-off point was selected as the value with the maximal sum of sensitivity and specificity. A logistic regression model was used to determine the predicated probability of the combination of miRNAs and US. The levels of miRNA in each group were defined by the median and interquartile range (IQR). All P-values were two-sided, and P<0.05 was considered to indicate a statistically significant difference.

Table I. miRNA-specific forward primer sequences.

Gene name	Primer sequence
miR-222	5'-GGGCTCAGTAGCCAGTGTAGATCC-3'
miR-221	5'-GGCGACCTGGCATACAATGTAGAT-3'
miR-146b	5'-GGCTGAGAACTGAATTCCATAGGC-3'
miR-21	5'-CAACACCAGUCGAUGGGCUGU-3'
miR-16	5'-GCTAGCAGCACGTAAATATTGGCG-3'

Results

Serum miRNA profiling in PTMC, BTN and control groups. We used qRT-PCR to measure the expression levels of serum miR-222, miR-221, miR-146b and miR-21 in patients with PTMC, BTN and the control subjects. The relative expression of miR-222, miR-221, miR-146b and miR-21 was significantly higher in patients with PTMC compared with BTN or in the control subjects (P<0.001 and P<0.001, respectively; Fig. 1).

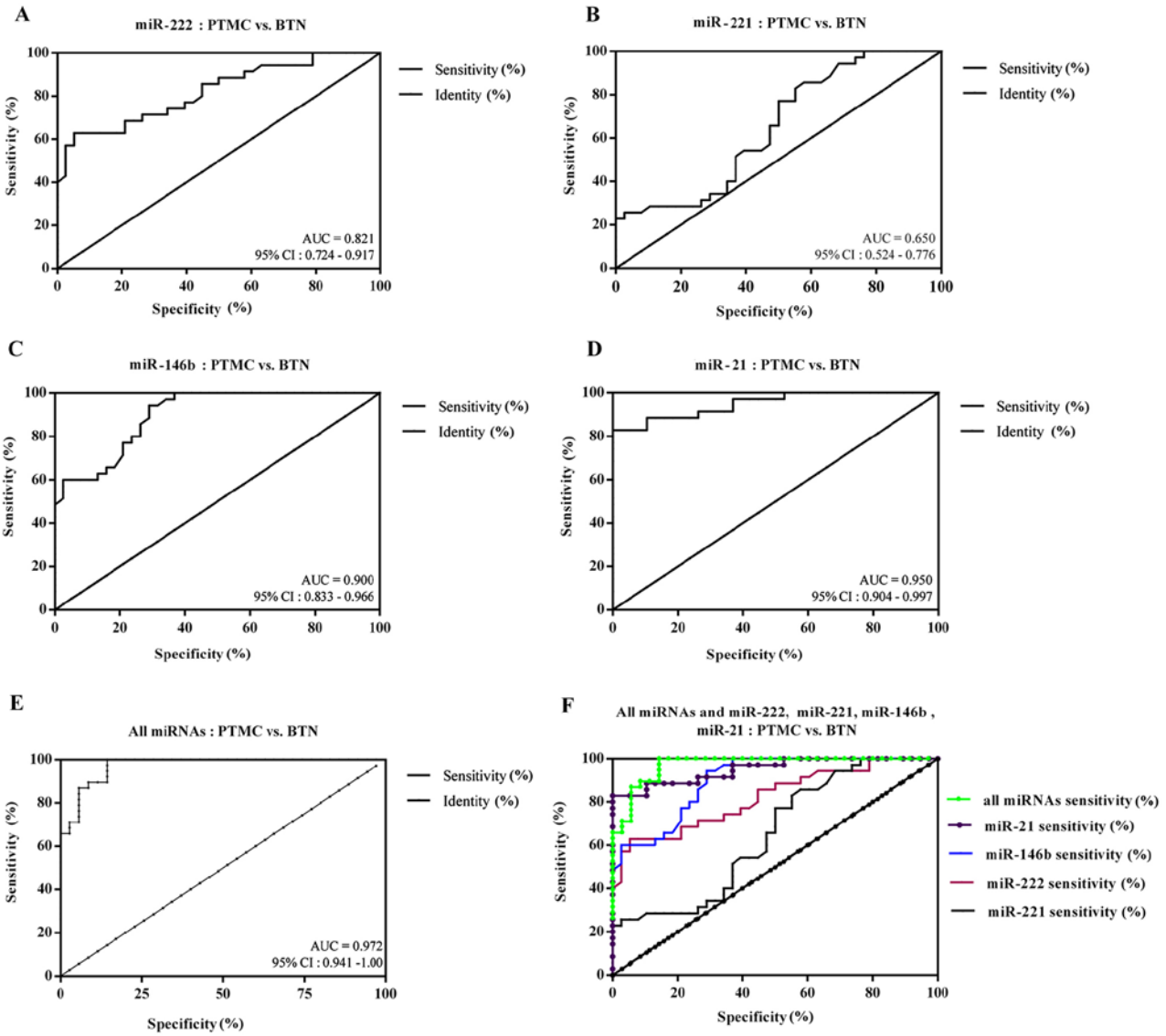

Figure 2. Diagnostic value of miRNAs in discriminating PTMC from BTN. ROC curves were used to distinguish among groups. (A) Serum miR-222 in PTMC vs. BTN. (B) Serum miR-221 in PTMC vs. BTN. (C) Serum miR-146b in PTMC vs. BTN. (D) Serum miR-21 in PTMC vs. BTN. (E) All four miRNAs (miR-222, miR-221, miR-146b and miR-21) in PTMC vs. BTN. (F) All four miRNAs and miR-222, miR-221, miR-146b and miR-21 in PTMC vs. BTN. PTMC, papillary thyroid carcinoma; BTN, benign thyroid nodules; ROC, receiver operating characteristic; AUC, area under the curve.

The fold-changes were 1.61, 2.26, 2.38 and 15.21, respectively, compared with the controls.

We performed ROC curve analysis of the predictive values of miRNAs for preoperative diagnosis in PTMC and BTN. The AUC of miR-222 was 0.821 (95% CI, 0.724-0.917, Fig. 2) with 60.53% sensitivity and 92.50% specificity, at a cutoff value of 1.59. The AUC of miR-221 was 0.650 (95% CI, 0.524-0.776, Fig. 2) with 77.14% sensitivity and 50% specificity, at a cut-off value of 2.37. The AUC of miR-146b was 0.900 (95% CI, 0.833-0.966, Fig. 2) with 77.14% sensitivity and 78.95% specificity, at a cutoff value of 1.74. The AUC of miR-21 was 0.95 (95% CI, 0.904-0.997, Fig. 2) with 88.57% sensitivity and 89.47% specificity, at a cut-off value of 6.06. Also, we analyzed the ROC curve of a combination of miR-222, miR-221, miR-146b and miR-21. The AUC of all 4 miRNAs was 0.972 (95% CI, 0.941-1.00, Fig. 2) with 86.84% sensitivity and 94.29% specificity, an improved sensitivity and specificity of diagnosis compared with each single miRNA.

Table II shows the sensitivity, specificity and accuracy of diagnosis with US, miRNAs and the combination of US and miRNAs in the detection of benign or malignant nodules in the thyroid. miR-222 had high specificity (P=0.003) and low sensitivity (no significance, P=0.375) and had a high false-negative rate and low false-positive rate compared with US. US combined with miR-222 had higher sensitivity (P<0.001) and specificity (no significance, P=0.377), and a low false-positive rate and false-negative rate compared with single US. US had a low sensitivity (no significance, P=0.205) and high specificity (P<0.001) and had a low false-positive rate and high false-negative rate compared with miR-221. US combined with miR-221 had high sensitivity and specificity (no significance, P=0.731 and P=0.109, respectively) and a low false-positive rate and false-negative rate compared with single US. US had a high sensitivity and low specificity (no significance, P=0.097 and P=0.552, respectively), as well as a high false-positive rate and low false-negative rate compared with

Table II. Comparison of US and miRNAs in the diagnostic method of PTMC.

Preoperative method of thyroid nodules	Specificity %	False-positive rate %	P-value	Sensitivity %	False-negative rate %	P-value
A, Comparison of US and miRNAs						
US	77.14	22.86	**0.003**	68.42	31.58	0.375
miR-222	92.5	7.5		60.53	39.47	
US	77.14	22.86	**<0.001**	68.42	31.57	0.205
miR-221	50	50		77.41	22.59	
US	77.14	22.86	0.097	68.42	31.57	0.552
miR-146b	86.84	13.16		62.86	37.14	
US	77.14	22.86	**<0.001**	68.42	31.57	**0.021**
miR-21	94.74	5.26		82.86	17.14	
US	77.14	22.86	**0.001**	68.42	31.57	**0.002**
All miRNAs	94.29	5.71		86.84	13.16	
B, Comparison of single US and the combination of miRNAs and US						
US	77.14	22.86	0.377	68.42	31.57	**<0.001**
miR-222+US	82.86	17.14		89.47	10.53	
US	77.14	22.86	0.731	68.42	31.57	0.109
miR-221+US	80	20		78.95	21.05	
US	77.14	22.86	**0.011**	68.42	31.57	**<0.001**
miR-146b+US	91.43	8.57		89.47	10.53	
US	77.14	22.86	**0.011**	68.42	31.57	**<0.001**
miR-21+US	91.43	8.57		94.74	5.26	
US	77.14	22.86	**0.048**	68.42	31.57	**<0.001**
All miRNAs+US	92.11	7.89		91.43	8.57	

P-values were calculated using Fisher's exact test; values in bold indicate significant differences. There was no significant difference noted in diagnostic specificity with US and miR-222, miR-21, miR-222 and all miRNAs (P=0.003, P<0.001, P<0.001 and P=0.001, respectively). The combination of US with miR-146b, miR-21 and all miRNAs was found to improve the specificity of diagnosis compared with the single US method (P=0.011, P=0.011 and P=0.048, respectively). There was significant difference noted in diagnostic sensitivity with US and miR-21 and all miRNAs (P=0.021 and P=0.002, respectively). The combination of US with miR-222, miR146b, miR-21 and all miRNAs was found to improve the sensitivity of diagnosis compared with the single US method (P<0.001, P<0.001, P<0.001 and P<0.001, respectively). US, ultrasound; PTMC, papillary thyroid microcarcinoma.

miR-146b. US combined with miR-146b had a high sensitivity and specificity (P=0.011 and P<0.001, respectively) and had a low false-positive rate and false-negative rate compared with single US. US had a low specificity and low sensitivity (P=0.021 and P<0.001, respectively), as well as a high false-positive rate and high false-negative rate compared with miR-21. US combined with miR-21 had a high sensitivity and specificity (P=0.011 and P<0.001, respectively) and a low false-positive rate and false-negative rate compared with single US. US had a low sensitivity and low sensitivity (P=0.001 and P=0.002, respectively), as well as a high false-positive rate and high false-negative rate compared with all miRNAs (combination of miR-222, miR-221, miR-146b and miR-21). US combined with all miRNAs had a high sensitivity and specificity (P=0.048 and P<0.001, respectively) and a low false-positive rate and false-negative rate compared with single US alone.

In addition, we performed ROC curve analysis to predict values of US for preoperative diagnosis of PTMC and BTN. The results indicated that the use of US had an AUC of 0.728 (95% CI, 0.609-0.846, Fig. 3) with 77.14% sensitivity and 65.79% specificity. We also analyzed miRNAs combined with US and evaluated whether the combination may improve the diagnosis of PTMC. The results indicated that the combination of US and miR-222 had an AUC of 0.866 (95% CI, 0.807-0.964, Fig. 3) with sensitivity 82.86% and specificity 89.47%. The combination of US and miR-221 had an AUC of 0.809 (95% CI, 0.725-0.893, Fig. 3) with sensitivity 80% and specificity 78.95%. The combination of US and miR-146b had an AUC of 0.942 (95% CI, 0.902-0.983, Fig. 3) with sensitivity 91.43% and specificity 89.47%. The combination of US and miR-21 had an AUC of 0.970 (95% CI, 0.933-1.0, Fig. 3) with sensitivity 91.43% and specificity 94.74%. The combination of four miRNAs and US indicated that the combination had an AUC of 0.971 (95% CI, 0.935-1.00, Fig. 3) with sensitivity 91.43% and specificity 92.11% and improved the diagnosis rate compared with US alone (Fig. 3).

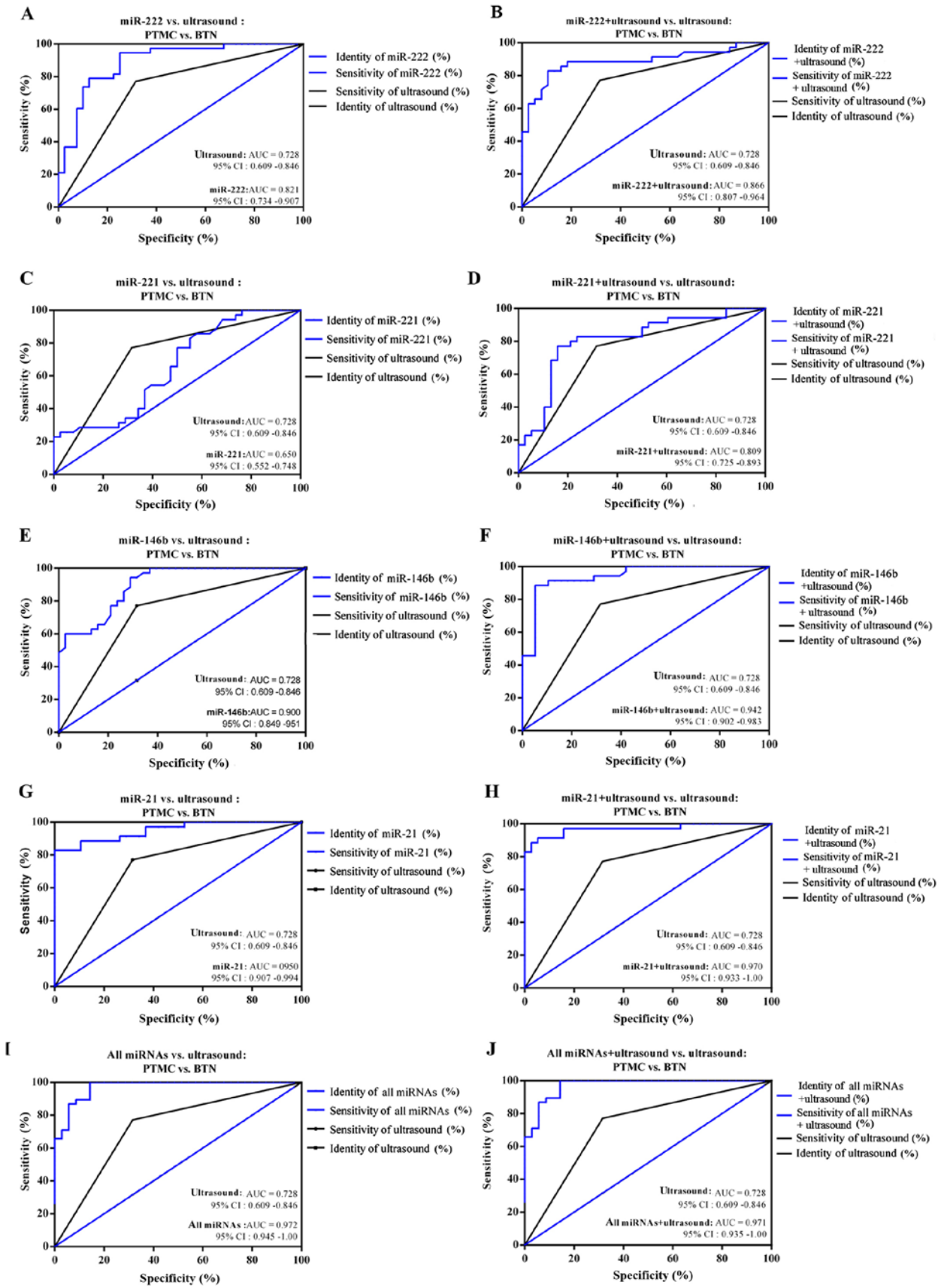

Figure 3. Diagnostic value of miRNAs in discriminating PTMC from and BTN. ROC curves were used to distinguish among groups. (A) Serum miR-222 and US in PTMC vs. BTN. (B) Combination of miR-222 and US compared with single US in PTMC vs. BTN. (C) Serum miR-221 and US in PTMC vs. BTN. (D) The combination of miR-221 and US compared with single US in PTMC vs. BTN. (E) Serum miR-146b and US in PTMC vs. BTN. (F) The combination of miR-146b and US compared with single US in PTMC vs. BTN. (G) Serum miR-21 and US in PTMC vs. BTN. (H) The combination of miR-21 and US compared with single US in PTMC vs. BTN. (I) All four miRNAs (miR-222, miR-221, miR-146b and miR-21) and US in PTMC vs. BTN. (J) The combination of all four miRNAs (miR-222, miR-221, miR-146b and miR-21) and US compared with single US in PTMC vs. BTN. PTMC, papillary thyroid carcinoma; BTN, benign thyroid nodules; ROC, receiver operating characteristic; AUC, area under the curve.

Table III. Characteristics of the patients with PTMC and PTC.

Characteristics	PTMC (n=58) n (%)	PTC (n=47) n (%)	P-value
Sex (male/female, %)			
Male, n (%)	20 (34.5)	18 (38.3)	0.420
Female, n (%)	38 (65.5)	29 (61.7)	
Age (years), n (%)			
<45	26 (44.8)	31 (66.0)	**0.024**
≥45	32 (55.2)	16 (34.0)	
Tumor location, n (%)			
Unilateral	32 (44.8)	24 (51.1)	0.698
Bilateral	26 (55.2)	23 (48.9)	
Multicentricity, n (%)			
Yes	22 (37.9)	12 (25.5)	0.211
No	36 (62.1)	35 (74.5)	
Extrathyroidal extension, n (%)			
Yes	10 (17.2)	8 (17.0)	0.346
No	48 (82.8)	39 (83.0)	
Metastatic lymph node, n (%)			
Yes	30 (51.7)	31 (66.0)	0.167
No	28 (48.3)	16 (34.0)	
TNM stage (AJCC), n (%)			
I/II	37 (63.8)	28 (59.6)	0.690
III/IV	21 (36.2)	19 (40.4)	
AMES, n (%)			
Low risk	51 (87.9)	42 (89.4)	0.535
High risk	7 (12.1)	5 (10.6)	
miRNAs, median (interquartile range)			
miR-222	1.83 (0.99-1.26)	2.69 (1.43-2.15)	**0.002**
miR-221	2.34 (1.59-3.10)	3.23 (1.79-5.39)	0.121
miR-146b	2.55 (1.39-3.97)	2.36 (1.79-4.26)	0.891
miR-21	13.69 (8.57-21.79)	34.06 (21.71-47.18)	**<0.001**

P-values were calculated using Fisher's exact test and Mann-Whitney U test results; values in bold indicate significant differences. Table representation of Mann-Whitney U test results, showing that the relative expression of miR-222, miR- 221, miR-146 and miR-21 is presented as the median and interquartile range ($2^{-\Delta\Delta Cq}$). TNM stage was assessed according to the AJCC staging systems; AMES is a risk stratification including age, metastasis, extent and size. PTC, papillary thyroid carcinoma; PTMC, papillary thyroid microcarcinoma; TNM, tumor node metastasis; AJCC, American Joint Committee on Cancer.

Correlation of miRNA expression in patients with PTMC and with PTC. A total of 105 newly diagnosed PTC patients, of which 58 were PTMC and 47 were non-PTMC with diameters >1 cm were investigated. There was a significant difference in terms of age between the two groups (P=0.024) and no significant differences for other clinical features including sex, tumor location, multicentricity, ETE, MLN, TNM staging or risk stratification (P>0.05, Table III). Both groups showed a female predominance. The results indicated that serum miR-222 and miR-21 levels were significantly higher in patients with PTC compared with PTMC (P=0.002 and P<0.001, respectively, Table III).

Correlation of miRNAs and clinicopathological characteristics of PTMC. We assessed the relationship between miRNA profiles and clinicopathological characteristics. The expression levels of the four miRNAs were not significantly different in regards to sex and age (P>0.05). The results indicated that serum miR-222, miR-146b and miR-21 levels were significantly higher in patients with bilateral lesions (P=0.005, P=0.001 and P=0.015, respectively, Table IV); patients with multifocal lesions (P=0.002, P=0.005 and P=0.005, respectively, Table IV); and patients with metastatic lymph nodes (P=0.014, P=0.031 and P=0.029, respectively; Table IV). The present results also indicated that serum miR-222 and miR-21 overexpression was clearly associated with ETE (P=0.026 and P=0.021, respectively; Table IV) and patients with a high risk (P=0.006 and P=0.026, respectively; Table IV). The present results also indicated that serum miR-222, miR-221 and

Table IV. Clinicopathogenetical features of the patients with PTMC and their correlation with circulating levels of the miRNAs.

Characteristics	No. of patients n (%)	Median (interquartile range) miR-222	P-value	Median (interquartile range) miR-221	P-value	Median (interquartile range) miR-146	P-value	Median (interquartile range) miR-21	P-value
PTMC	58 (100)	1.83 (1.43-2.15)		2.34 (1.59-3.10)		2.36 (1.79-4.26)		13.69 (8.57-21.79)	
Age (years)									
<45	26 (44.8)	1.87 (1.31-2.94)	0.544	2.71 (1.71-3.44)	0.255	1.99 (1.25-3.82)	0.063	11.59 (6.85-15.786)	0.515
≥45	32 (55.2)	2.16 (1.46-3.84)		2.17 (1.56-2.76)		4.25 (2.14-5.32)		12.58 (7.73-29.98)	
Sex									
Male	20 (34.5)	1.87 (1.43-3.37)	0.974	2.58 (1.53-3.10)	0.974	1.41 (1.15-5.48)	0.274	8.79 (6.29-17.04)	0.190
Female	38 (65.5)	1.96 (1.32-3.03)		2.29 (1.69-3.36)		3.16 (2.02-4.84)		13.36 (8.46-23.69)	
Tumor location									
Unilateral	32 (44.8)	1.62 (1.20-2.41)	**0.005**	2.34 (1.53-3.17)	0.626	1.89 (1.23-4.26)	**0.001**	10.09 (6.53-13.88)	**0.015**
Bilateral	26 (55.2)	2.92 (1.87-5.18)		2.29 (1.82-3.16)		4.59 (2.59-5.71)		18.48 (10.92-34.19)	
Multifocation									
Yes	22 (37.9)	3.47 (2.69-5.26)	**0.002**	2.38 (1.74-4.11)	0.562	4.69 (3.78-5.48)	**0.005**	29.45 (11.79-35.81)	**0.005**
No	36 (62.1)	1.64 (1.27-2.00)		2.30 (1.57-2.95)		2.10 (1.29-4.20)		10.63 (7.27-15.45)	
Extrathyroidal invasion									
Yes	10 (17.2)	4.93 (180-5.38)	**0.026**	2.38 (2.10-4.43)	0.198	4.30 (2.52-5.50)	0.066	25.02 (13.62-45.42)	**0.021**
No	48 (82.8)	1.83 (1.34-2.81)		2.20 (1.54-3.00)		2.20 (1.30-4.79)		10.63 (6.93-15.67)	
Metastatic lymph node									
Yes	30 (51.7)	2.34 (1.60-3.44)	**0.014**	2.38 (1.69-3.58)	0.371	3.75 (2.02-5.48)	**0.031**	14.41 (9.10-29.10)	**0.029**
No	28 (48.3)	1.28 (1.11-1.93)		2.18 (1.56-2.71)		1.72 (1.15-4.11)		8.14 (6.26-11.31)	
TNM stage (AJCC)									
I/II	37 (63.8)	1.66 (1.30-2.36)	**0.001**	2.14 (1.54-2.70)	**0.004**	2.32 (1.30-4.63)	0.104	10.09 (6.40-16.81)	**0.008**
III/IV	21 (36.2)	5.11 (2.69-5.54)		4.56 (2.22-8.25)		4.59 (2.27-5.49)		17.79 (12.97-37.43)	
AMES									
Low risk	51 (87.9)	1.76 (126-2.75)	**0.006**	2.25 (1.54-2.97)	0.191	2.18 (1.76-4.63)	0.129	10.63 (7.10-15.56)	**0.026**
High risk	7 (12.1)	4.95 (2.23-5.44)		2.64 (2.09-5.03)		2.46 (2.08-3.78)		26.53 (13.77-53.41)	

Table representation of Mann-Whitney U test results, showing that the relative expression of miR-222, miR-221, miR-146 and miR-21 is presented as the median and interquartile range ($2^{-\Delta\Delta Cq}$). TNM stage was assessed according to International Union Against Cancer (UICC) staging systems; AMES is a risk definition including age, metastasis, extent and size. The low-risk group was defined as those patients who were less than 45 years of age and had stage I PTC and those patients who were aged 45 years or more with stage I and II PTC. The remaining patients were defined as high-risk group. P-value was determined by Mann-Whitney U test; values in bold indicate significant differences. AJCC, American Joint Committee on Cancer; PTC, papillary thyroid carcinoma; PTMC, papillary thyroid microcarcinoma; SD, standard deviation; TNM, tumor-node-metastasis.

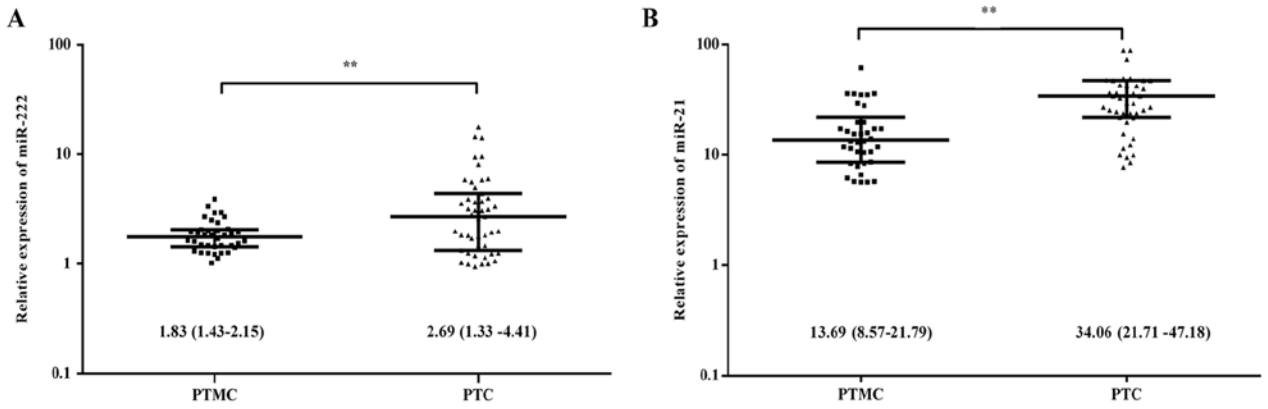

Figure 4. Diagram of the Mann-Whitney U test results, showing that the relative expression levels of miR-222 (A) and miR-21 (B) in the PTC group (n=47) were significantly higher than levels in PTMC (n=58, **P<0.01). The relative expression of miRNAs is presented as the median and interquartile range $2^{-\Delta\Delta Cq}$. PTMC, papillary thyroid microcarcinoma; PTC, papillary thyroid microcarcinoma.

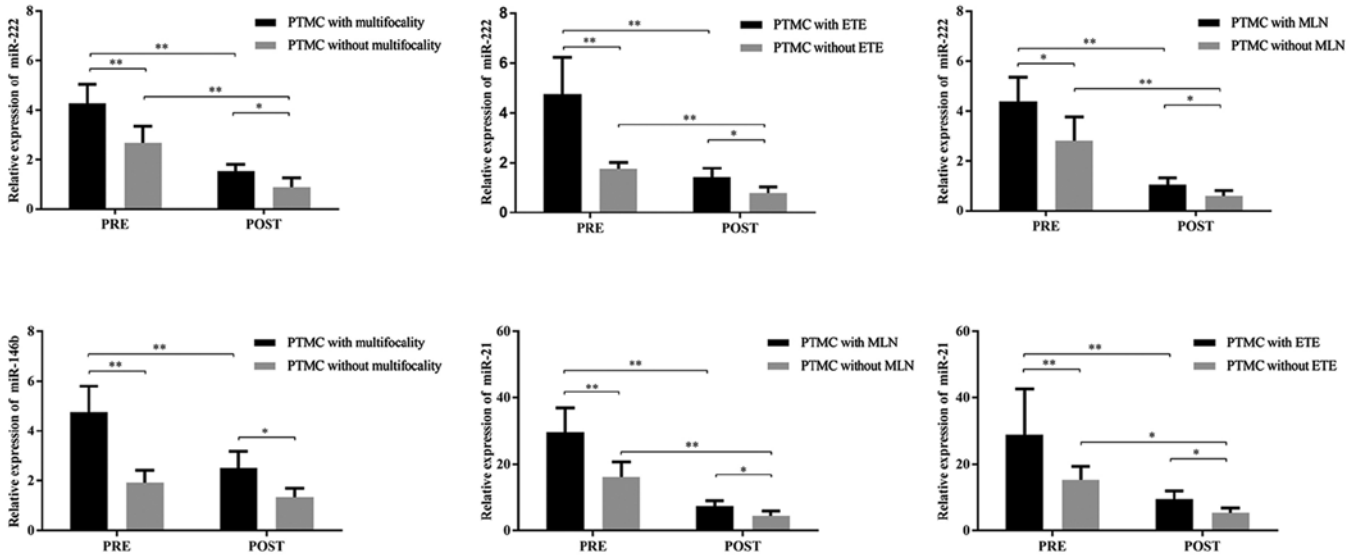

Figure 5. Expression of four miRNAs in patients with PTMC (n=30) before and after surgery in the various groups divided into two groups according to prognostic factors of PTMC. The levels of miR-222, miR-146b and miR-21 were significantly higher in patients with poor prognoses before and after surgery (**P<0.01, *P<0.05). The relative expression of miRNAs is presented as the median and interquartile range $2^{-\Delta\Delta Cq}$. All P-values were determined using the Kruskal-Wallis H test and Scheffe's Post Hoc multiple comparisons test. PTMC, papillary thyroid microcarcinoma; PRE, preoperative; POST, postoperative, miR, microRNA; ETE, extrathyroidal extension; MLN, metastatic lymph nodules.

miR-21 overexpression was clearly associated with advanced TNM stage (P=0.001, P=0.004 and P=0.008, respectively; Table IV).

Association of miRNA expression and poor prognostic factors before and after surgery in PTMC. We also measured the miRNA expression levels one month after surgery and compared them with preoperative expression in serum from patients with PTMC. The results suggested that the serum expression of miR-222, miR-221, miR-146b and miR-21 decreased significantly after surgery compared with before surgery in 30 patients with PTMC (P<0.001, P=0.002, P=0.013 and P<0.001, respectively, Fig. 4).

Simultaneously, we analyzed the expression levels of the four miRNAs in various groups according to the prognosis of PTMC patients before and after surgery. The results demonstrated that the miR-222 expression levels were significantly higher in patients with multifocal lesions, ETE and MLN before (P<0.001, P<0.001 and P=0.012, respectively) and after (P=0.032, P=0.031 and P=0.014, respectively) surgery (Fig. 5). miR-146b expression was significantly higher in patients with multifocal lesions before (P<0.001) and after (P=0.016) surgery (Fig. 5). miR-21 expression was significantly higher in patients with ETE and MLN before (P=0.002 and P<0.001, respectively) and after (P=0.027 and P=0.035, respectively) surgery (Fig. 5). There was no difference in the other groups (P>0.05).

Discussion

Ultrasound (US) has attracted considerable research attention in the diagnosis of papillary thyroid microcarcinoma (PTMC) as a non-invasive imaging method that is useful for identifying benign or malignant nodules. However, US has limitations

associated with small nodules, especially those smaller than 5 mm, and has a low sensitivity and specificity of diagnosis of PTMC. The proportion of non-diagnostic and indeterminate US accounted for 20-30% in PTMC, especially for nodules smaller than 5 mm in maximal diameter. Many of these patients repeatedly undergo fine-needle aspiration (FNA) or surgery, but fewer than 20% are found to be malignant by pathology (11,37). There is no doubt that repeated FNA can increase the cost and psychological burden of patients. In addition, US is not sensitive for the detection of central lymph node metastasis, detecting <10% (38). This is mainly due to the location of the nodes as well as interference from the trachea and pulsating vessels. FNA is recommended for thyroid nodules larger than 10 mm, even for those with highly suspicious sonographic features, according to the 2015 ATA guidelines (20). Therefore, US-guided FNA is not widely appropriate to diagnose thyroid nodules, especially for small thyroid nodules or multifocal lesions.

miRNAs can be isolated from serum or plasma, and they can be used to detect many types of cancers (39-41). However, the promising use of miRNAs as biomarkers for PTC has been reported in only a few studies (28,35,42-45). To date, no studies have examined circulating miRNA expression in PTMC patients, and none have attempted to use serum miRNAs to analyze their association with the clinicopathologic features of PTMC. Although a few previous studies have demonstrated that circulating let-7e, miR-222, miR-221, miR-146b, miR-151-5p, miR-190 and miR-95 are overexpressed in PTC compared with benign thyroid nodules and healthy controls and offered a promising method for preoperative diagnosis of PTC, there is controversy and unresolved issues involving these studies (28,35,42-45). Firstly, different expression levels of the target miRNAs were noted in these various studies. These findings could be interpreted as indicating that differential expression of miRNAs is due to different regions and races of patients with PTC. Secondly, the expression patterns of serum miRNAs have not yet been clearly elucidated with patterns of PTMC in the current studies. Thirdly, the correlation between several miRNAs and their link to a poor prognosis remains ambiguous. Finally, a comparative analysis of the sensitivity and specificity of diagnosis with US and miRNAs has not yet been performed for distinguishing PTMC from benign thyroid lesion (BTN).

In the present study, we performed an initial analysis of the expression of circulating miRNAs in patients with PTMC or BTN and in control subjects and estimated their diagnostic usefulness for distinguishing between benign and malignant lesions. The expression of tissue-derived miR-222, miR-221, miR-146b and miR-21 was increased in PTC compared with BTN, and their overexpression in tissue was associated with a poor prognosis based on the results of the published literature. Therefore, we selected 4 miRNAs, including miR-222, miR-221, miR-146b and miR-21, as target miRNAs. Our results indicated that serum miR-222, miR-221, miR-146b and miR-21 are overexpressed in patients with PTMC compared with BTN and control subjects. In addition, our results indicated that the relative expression levels of miR-222 and miR-21 in the PTC group are significantly higher than levels in PTMC. For miR-221 and miR-146b, there was no significant difference in expression between the PTMC and PTC groups. The difference in expression is currently unexplained. Kim *et al* analyzed the gene expression profiles of PTMC and PTC. Their results indicated that for gene expression profiles of PTMCs and PTCs, no significant difference was found. Therefore, they proposed that PTMC should not be considered to be a simple occult indolent thyroid cancer, but as an earlier stage of the disease that eventually evolves into PTC, since the gene expression profiles of PTMCs were not different from those of PTCs (46).

We also used ROC curves to verify their value in the diagnosis of PTMC compared with BTN before surgery. The ROC of miR-222, miR-146b and miR-21 had a higher sensitivity and specificity for diagnosis compared with US in separating the PTMC from the BTN groups. In addition, the ROC of the combination of miRNAs and US had higher sensitivity and specificity for diagnosis compared with single US or miRNAs in separating the PTMC from BTN groups. Therefore, our results indicated that the combination of US and serum miR-222, miR-221, miR-146b and miR-21 may serve as a preoperative invasive biomarker of diagnosis and differential diagnosis. Furthermore, the levels of the four miRNAs were significantly decreased one month after surgery compared with before surgery in serum from patients with PTMC. This finding could suggest that circulating miRNAs are closely associated with the tumorigenesis of PTMC.

Although increasing numbers of studies have demonstrated that circulating miRNAs have been examined in bodily fluids including plasma, urine, peripheral blood and saliva, they were found to be quite stable and reproducible in serum. However, the pathogenesis involved in the release of miRNAs from tumor tissue into the circulation remains unclear. There was some speculation that circulating miRNAs may be a product of tumor cell death and lysis, or they may be released from tumor-derived macrovesicles or exosomes, or they may be a product of a cancer-associated immune response (47,48).

Although PTMC generally has an excellent prognosis, the long-term rate of recurrence of PTMC has been reported to be as high as 10% (49). A meta-analysis including more than 4,000 patients with PTMC indicated that 28% of patients had metastatic lymph nodes, 3.3% of patients progressed to recurrence, and 0.6% of tumors had a distant metastasis (50). Therefore, it is important to identify predictive factors associated with a poor prognosis that may help physicians adopt tailored appropriate treatment strategies for patients.

Currently, genetic biomarkers are used to evaluate aggressive behaviors in PTMC. Several reports have suggested a correlation between BRAF mutations and advanced TNM stage, extrathyroidal extension (ETE), metastatic lymph nodules and multifocal lesions (51-53). However, BRAF mutations have been found in 24-63% of PTMC cases and there is no large set of data reporting an association between BRAF and aggressive behaviors of PTMC (54-57). Currently, there are no reliable clinical features including molecular markers, that can differentiate PTMC in patients who develop progressive disease from indolent PTMC (58-61). Further studies are needed not only in regards to the natural history of PTMC but also concerning the identification of markers indicating progressiveness or indolence in PTMC.

In this study, we assessed the relationship between miRNA profiles and clinicopathological characteristics. The

results indicated that serum miR-222, miR-146b and miR-21 levels were significantly associated with factors indicating a poor prognosis, including those with bilateral and multifocal lesions, metastatic lymph nodes and high-risk groups. Previous studies have documented that miR-222, miR-221, miR-146b and miR-21 are significantly overexpressed in tumor specimens from aggressive and recurrent PTC in tumor specimens (29,31,62-64). Chou *et al* demonstrated that the expression levels of miR-221, miR-222, and miR-146b were significantly associated with ETE and that the expression levels of miR-221 and miR-146b were significantly higher in the high-risk PTC group (31).

The miR-146b expression levels in PTCs with BRAF mutations were significantly higher than in those without mutations. However, it is essential to identify the gene targets of these miRNAs (31). Previous studies have already demonstrated that miR-146b is overexpressed in tumor specimens from PTC patients and is associated with a poor prognosis or aggressive behavior, including extrathyroidal extension, advanced stages, BRAF mutations, large tumor size and shorter survival, thus it was regarded as a relevant diagnostic and prognostic marker for PTC. miR-146b miRNA is the product of the miR-146b gene that is located on chromosome 10 at position q24.32. Other studies that have focused on various computational target genes in PTC identified 34 target genes for miR-146b (65). The analysis of their biological activities showed that many target genes were involved in cellular proliferation, differentiation, apoptosis, the cell cycle, and signaling transduction pathways, suggesting a role of this miRNA in the pathogenesis of PTC (65).

In addition, similar to previous reports (18,45,66-68) our results indicated that the expression levels of miR-222 and miR-221 are significantly increased in serum from III/IV stage PTMC cases. A previous study suggested that both miR-222 and miR-221 are clustered on the X chromosome, and it is likely that they are encoded by a single polycistron, as was previously suggested (69). They are involved in cell proliferation through the inhibition of the cell cycle regulator, p27kip1, in human papillary carcinomas. A study by Mardente *et al* showed that HMGB1 increased the expression of miR-221 and miR-222 in primary cultures of excised papillary lesions. The overexpression of oncogenic miR-221 and miR-222 caused by HMGB1 was found to be associated with an increase in malignancy scores (70).

In our analysis, we demonstrated that miR-21 expression levels were significantly increased in the serum of PTMC patients with various factors predictive of a poor prognosis, including bilateral and multifocal lesions and metastatic lymph nodules. Previous studies have reported that upregulation of miR-21 is associated with various metastatic cancers, including prostate, colon, bladder, lung, breast, esophageal and PTC (29,71-74). In addition, miR-21 is associated with aggressive behaviors and poor survival in some cancers (75-78). Among miR-21 targets that could have a role in PTC metastasis, intercellular cell adhesion molecular-1 (ICAM1) stands out (79). In fact, it is upregulated in PTC, and its expression is correlated with aggressive tumor features such as lymph node metastasis (80). The downregulation of miR-21 in recurrent PTC is therefore in agreement with the reported upregulation of ICAM1 in aggressive PTC. It has been shown that miR-21 participates in the process of cell division, differentiation and apoptosis in oncogenesis (29,81-83).

In order to further confirm the correlation between miRNA expression and poor prognosis, we divided the patients into two groups based on the clinicopathological features associated with poor and good prognoses in postoperative patients with PTMC. The results indicated that miRNA expressions levels were significantly higher in patients with multifocality, ETE and metastatic lymphocyte nodules compared with the matched groups with a good prognosis after surgery. These results further confirmed that expression of miRNAs is significantly associated with tumorigenesis and a poor prognosis in PTMC.

Finally, although circulating miRNAs have been confirmed as molecular markers of diagnosis and poor prognosis in PTMC, there were several controversial issues. Firstly, the identified miRNA expression patterns reported in various studies were not completely identical in PTC, which may be due to differences in regions and race, the heterogeneity of PTC or environmental factors. Secondly, the exact mechanism behind circulating miRNAs and the tumorigenesis of PTMC remains unknown. Therefore, it is important to elucidate further the mechanism of action of miRNAs in PTMC. Thirdly, our study was limited due to the fact that we included a cohort of patients from a single center and that the sample size was small. Finally, we also needed longer-term follow-up for patients to obtain relatively accurate results.

In conclusion, miRNAs could serve as biomarkers to distinguish between malignant and benign thyroid nodules. The combination of miRNAs and US could improve the sensitivity and specificity of diagnosis. miRNAs were found to be significantly associated with a poor prognosis of patients with PTMC and could be used as prognostic molecular markers for patients with PTMC before and after surgery. This study results suggest that circulating miRNAs may be useful as non-invasive molecular biomarkers of diagnosis and prognosis for PTMC, but this should be confirmed with continued follow-up and verified with larger patient samples.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding authors on reasonable request.

Authors' contributions

YZ, HQ and DX acquired the data and created a draft of the manuscript; YZ, JP and ZY prepared the experimental

materials and performed the assays; ZY, DX and JS interpreted data, performed the statistical analysis and analyzed the results; LS, HQ and YW revised and approved the final version of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was granted ethical approval by the Institutional Review Board of Harbin Medical University. Written informed consent was obtained from all participants involved in the study. The reference code for the ethical approval was no. HMUIRB20150020.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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