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miR-146b measurement in FNA to distinguish papillary thyroid cancer from benign thyroid masses

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Thyroid nodules are present in 5–10% of adults on physical palpation of the thyroid gland; it is much higher on thyroid ultrasonography-up to 50–70% in people older than 60 years [1]. One of the main diagnostic problems in the thyroid field involves the preoperative assessment of thyroid nodules. Palpable nodules are common in the adult population, with an estimated prevalence in the United States in the range of 4–7% or 10–18 million affected individuals [2]. Fine-needle aspiration (FNA) is an important method for preoperative evaluation of thyroid nodules, although in 10–20% of samples, the precise diagnosis is unclear, so many are reported as indeterminate or atypical [3]. Most of these patients undergo surgery, although only 8–17% of surgically removed nodules are found to be malignant [2]. Improvement in diagnostic accuracy can be achieved by additional testing of the FNA material for somatic mutations known to occur in thyroid tumours, although its sensitivity is limited because most papillary and follicular cancers have no known mutations [4]. Hence, there is an urgent need for identifying biomarkers that can be used as an adjunct to FNA to distinguish benign thyroid nodules from papillary thyroid cancer (PTC).

miRNAs are a class of small noncoding RNAs involved in a wide range of processes such as proliferation, development, apoptosis and metabolism. Microarray miRNAs profile expression analysis in thyroid cancer found aberrant expression of several miRNAs in PTCs. Serum miR-124 has value as a diagnostic biomarker in pancreatic cancer, where levels may have prognostic value, and in osteosarcoma, upregulation of miR-21 is associated with poor clinicopathological characteristics [5,6]. In patients with PTC, low miR-199a-3p expression levels are linked to tumour, node and metastases (TNM) stage, extra-thyroidal extension, lymph node metastasis, distant metastasis and recurrence of lymph node metastasis [7]. Several lines of evidence indicate that aberrant miR expression profiles can differentiate thyroid cancers from benign thyroid lesions and normal thyroid tissue, suggesting that miRs may have potential use as a diagnostic tool in thyroid pathology [8,9]. A differential overexpression of miR-146b has been observed in different types of cancer, including PTC [10]. The high expression of miR-146b in PTC has been positively correlated with the malignancy and aggressiveness in clinicopathological correlative studies [11]. In addition, higher miR-146b expression levels had significantly poorer overall survival compared with those with lower miR-146b levels [10]. Up-regulation of tissue miR-146a expression relates to carcinogenesis and deterioration of PTC, so that it may act as potential biomarkers for PTC patients [12].

We hypothesised that miR-146b in FNA can distinguish PTC from benign thyroid masses, and explored possible implications of miR-146b identification on the initial surgical treatment of thyroid nodules with PTC cytology. In addition, we probed links between miR-146b expression and key clinicopathological indicators, and so evaluated the potential usefulness of miR-146b in predicting invasion and metastasis in PTC.

The Committee on Human Research at the Affiliated Hospital of Qingdao University approved the study. Following informed consent, thyroid tissue samples and clinical and histopathology data were obtained from 336 patients being investigated for suspected thyroid disease at the Department of Thyroid Surgery, Affiliated Hospital of Qingdao University. All patients had FNA before surgery either by direct puncture after palpating the thyroid nodule, or more commonly under ultrasound guidance.

Part of the biopsy samples was used for FNA before surgery. Briefly, nodules were aspirated 3 to 5 times with a 22 or 25-gauge needle. From each FNA pass 1–3 smears

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were prepared and fixed in alcohol for Papanicolaou staining and air dried for Giemsa staining. Liquid-based preparation can also be made after a FNA pass, with the needle rinsed in normal saline or ThinPrep solutions. As a result, 3 to 15 glass slides from each patient were taken and examined, as either Giemsa- or Papanicolaou-stained slides. Regardless the staining method used, all slides with diagnostic material were used for evaluation and clarification of each case. In addition, part of the biopsy samples were immediately stored at −80 °C until RNA extraction.

Purification of miRNA was performed by using miRNeasyMini Kit (Qiagen, Valencia, CA, U.S.A.). Quantitative reverse transcription (RT) was performed using miScript II RT Kit. cDNA generated from the miScript II RT Kit was used as a template for real-time PCR with the miScript SYBR Green PCR Kit with miRNA-specific primers for miR-146b (Qiagen). qPCR was run on a Rotor-Gene 6000 (Corbett, Life Science, Sydney, Australia): 1 cycle at 95 °C for 15 min, 40 cycles at 94 °C for 15s, 55 °C for 30s, and 70 °C for 30s. After 40 cycles, a melting curve was generated by slowly increasing (0.1 C/s) the temperature from 55 °C to 99 °C, while measuring fluorescence. Samples were tested in triplicate and relative expression levels calculated using U6 small nuclear RNA (U6) (SNORD61, Qiagen) as the endogenous control.

Data are presented as median (IQR). Kruskal-Wallis and Mann-Whitney tests were used to determine differences of miR-146b level in FNA of PTC from benign thyroid masses. Categorical data was compared by chi-squared testing. Sensitivity and specificity of miR-146b was determined based on the histological diagnosis. The area under the receiver operating characteristic (ROC) curve (AUC) was estimated. $P < 0.05$ was considered significant.

Tissue diagnoses were confirmed by histology, the gold standard to determine accuracy. Clinicopathologic variables included sex, age, tumour size, encapsulation, multicentricity, lymph node metastasis and distant metastasis. Cancer stage was determined according to the TNM staging system of the seventh edition of the *AJCC Cancer Staging Manual*.

Histological diagnoses were primary PTC ($n = 246$), benign thyroid nodules ($n = 90$). The clinicopathological characteristics of the patients are shown in Table 1. Of the PTC patients, 192 were at TNM stage I/II, 50 were at TNM stage III/IV. Of patients with benign thyroid nodules, 62 had a thyroid adenoma and 28 had a classical nodular goiter. PTCs and benign thyroid nodules patients were age (49.7 years [16.4–70.4] and 49.3 [19.4–78.4], $p = 0.439$) and sex (M:F = 54:192 and M:F = 28:62, $P = 0.215$) matched. Relative expression of miR-146b compared to the general U6 mRNA level was significantly higher in malignant lesions compared with benign lesions (194.3 [IQR 1.4–392.4] vs. 18.7 [0.9–113.6 ng/mL] $p = 0.0013$). Levels of miR146b were significantly higher in PTCs with advanced TNM stage, lymph node metastasis and distant metastasis (Table 1).

FNA diagnosed 198 cases with PTC, 8 with medullary thyroid cancer, 8 with follicular thyroid carcinoma, 7 with thyroid follicular adenoma, 2 with anaplastic thyroid cancer, 13 with benign thyroid masses and 10 with indeterminate or suspicious PTC. Of the 90 benign thyroid nodules, FNAs diagnosed 10 cases of PTC, 2 medullary thyroid cancers, 2 follicular thyroid carcinomas, 70 benign thyroid masses and 6 with indeterminate or suspicious benign masses. Thus, the accuracy of FNA for PTCs was 80.5% (198/246), the sensitivity was 76.9%, specificity was 86.4%, positive predictive value (PPV) was 85.3%, and negative predictive value (NPV) was 94.6%.

In order to test the potential use of miR-146b on classifying FNA specimens, ROC curve analysis was performed (Figure 1). The AUC of miR-146b was 0.87 (95%CI: 0.76–0.97) (cutoff value 45.6, sensitivity 92.1%, specificity 82.3%). Of the 246 PTCs, miR-146b was >45.6 in 223 cases. Of the 90 benign thyroid masses, miR-146b was >45.6 in 9 cases. Thus, the accuracy of a relative expression of 45.6 miR-146b 'units' for PTCs was 90.6% (223/246), PPV was 93.3%, NPV was 96.2%. Of the 198 cases of FNA-PTCs, miR-146b was >45.6 in 192 cases, so the accuracy was 97%.

The present study confirms that FNA is an effective tool in identifying patients with PTCs and benign thyroid masses. In our study, the accuracy, sensitivity, specificity, PPV and NPV of FNA for PTCs compare favourably to those reported in other series [13]. In our study, 20% of all PTC patients are least accurately identified by FNA. In addition, 2/9 of patients of benign thyroid masses are

Table 1. Associations between miR-146b level and clinicopathologic factors in PTCs.

Clinicopathological feature	Number	miR-146b	*p*-value
Sex		[median (IQR)]	0.274
Male	54	188.4(1.41–389.2)	
Female	192	195.3(1.24–381.6)	
Age (year)			0.423
≤50	170	192.6(1.38–406)	
>50	76	197.9(1.39–398.2)	
TNM stage (AJCC)			0.018
I + II	192	164.3(1.9–258.3)	
III + IV	54	283.8(7.8–667.2)	
Tumour size (cm)			0.098
≤1	99	190.7(1.46–391.2)	
1<,<2	79	195.6(1.5–410.2)	
≥2	68	217.4(1.39–444.5)	
Encapsulation			0.452
Yes	74	196.5(1.6–487.3)	
No	172	193.8(1.4–514.6)	
Multicentricity (>2)			0.304
Yes	138	191.5(1.5–398.7)	
No	108	194.3(1.4–412.3)	
Lymph node metastasis			0.04
Yes	187	217.5(2.3–587.6)	
No	59	179.4(1.5–301.4)	
Distant metastasis			0.002
Yes	27	258.7(34.6–876.3)	
No	219	164.3(1.2–289.4)	

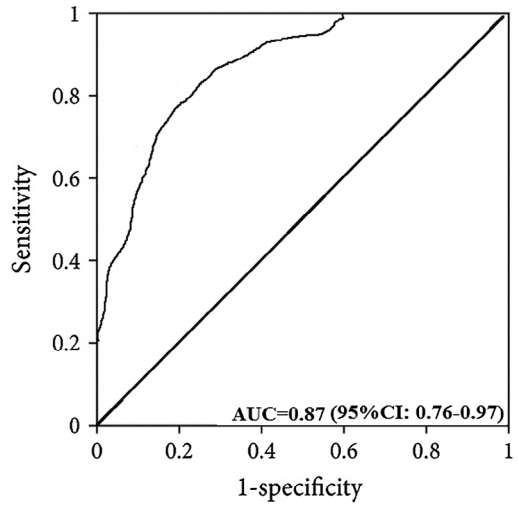

Figure 1. Receiver operator characteristic (ROC) curve for predicting PTCs according to the miR-146b levels.

least accurately identified by FNA. It is clearly this subset of patients in which the greatest need exists for more reliable predictors of PTC and benign thyroid masses.

In recent years, advances in the analysis of miR expression profiles have been made in various human cancers, including thyroid cancer [7,14]. Other studies have indicated that an aberrant miR expression profile can separate PTC from normal thyroid tissue and benign thyroid lesions. Chen et al. [9] measured six miRs (miR-146b, -221,-222, -146a, -155, and -187) in 20 PTC FNA samples and 20 benign thyroid lesions, and found only miR-146b, -221, and-222 to be able to differentiate PTC from benign lesions.

We sought to further evaluate the diagnostic utility of miR-146b in thyroid FNA cytology. Our data indicate that as miR-146b showed significant increased expression between malignant and benign lesions, it has strong potential for better diagnostic performance. The difference of accuracy between FNA and miR-146b may reflect that miR-146b has relatively strong diagnostic value for PTC. We also found that increased miR-146b expression in PTC specimens with advanced disease stage and indicated that it may be a useful prognostic marker for PTC. In addition, we have shown that increased miR-146b expression in PTC cells was linked to metastases to regional lymph nodes or distant organs, further noting that increased miR-146b is involved in PTC invasion and metastasis.

This work represents an advance in biomedical science because it shows that miR-146b may be used to improve the diagnostic accuracy of FNA biopsy and to distinguish PTCs from benign thyroid masses, and may be a potential target for therapeutic intervention.

Disclosure statement

No potential conflict of interest was reported by the authors.

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