Circulating MicroRNA Profiles as Potential Biomarkers for Diagnosis of Papillary Thyroid Carcinoma

Shuang Yu,* Yuanyuan Liu,* Jingsong Wang, Zhuming Guo, Quan Zhang, Fengyan Yu, Yunjian Zhang, Kai Huang, Yanbing Li, Erwei Song, Xi-long Zheng, and Haipeng Xiao

Departments of Endocrinology (S.Y., Y.Liu, Y.Li, H.X.) and Vascular Surgery (J.W., Y.Z., K.H.), The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China 510080; Department of Head and Neck Surgery (Z.G., Q.Z.), Cancer Center, Sun Yat-sen University, Guangzhou, China 510060; Department of Breast Surgery (F.Y., E.S.), Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China 510120; and Department of Biochemistry and Molecular Biology (X.Z.), University of Calgary, Calgary, Alberta, Canada T2N 4N1

Context: There are no known effective and reliable biomarkers to distinguish benign thyroid nodules from papillary thyroid carcinomas (PTC). Previous studies have indicated that serum microRNA (miRNA) profiles may be diagnostic and/or prognostic markers for numerous other cancers.

Objective: We studied circulating miRNA profiles in patients with PTC or benign nodules and healthy controls to identify serum miRNA that may be useful as markers for PTC.

Design, Setting, and Participants: Genome-wide serum miRNA expression profiles were determined using Solexa sequencing followed by extensive quantitative RT-PCR validation in 245 subjects (106 patients with PTC, 95 patients with benign nodules, and 44 healthy controls). A panel of miRNA was used to assess the expression of specific miRNA in the sera and thyroid tissues of patients with PTC or benign nodules.

Results: The expression of serum let-7e, miR-151-5p, and miR-222 was significantly increased in PTC cases relative to benign cases and healthy controls. Receiver operating characteristic curve analyses indicated that use of these three miRNA had a high diagnostic sensitivity and specificity for PTC. Serum let-7e, miR-151-5p, and miR-222 levels were found to be well correlated with certain clinicopathological variables, such as nodal status, tumor size, multifocal lesion status, and Tumor-Node-Metastasis stage. Expression of serum miR-151-5p and miR-222 in a subset of PTC patients decreased significantly after tumor excision. Increased expression of miR-151-5p and miR-222 was also found in the tissue of PTC patients.

Conclusions: Our study demonstrates that serum miRNA profiles may be used as novel and minimally invasive diagnostic markers for PTC. (J Clin Endocrinol Metab 97: 2084–2092, 2012)
plasm (4). However, the results of this procedure may show suspicious malignancy, suspicious follicular neoplasm, or features of atypia of undetermined significance in the diagnosis of thyroid nodules. Most cases (60–75%) of suspicious papillary carcinoma are proved to be PTC and the rest mostly follicular adenomas (5). Moreover, inadequate FNA sampling contributes to nondiagnostic or unsatisfactory cytology, in which case, a repeat aspiration is recommended (6). FNA is an invasive diagnostic method and heavily dependent on the technical performance and experience of the operators. Taken together, all of the above creates a gap in the clinical decision pathway and an additional burden in patients with thyroid nodules (7). Thus, considerable effort has been made to identify other reliable markers for primary PTC.

MicroRNA (miRNA) are a recently identified class of small, endogenous, noncoding RNA that act as negative regulators of gene expression. miRNA can impact cell growth, differentiation, apoptosis, and adhesion, all fundamental cellular processes implicated in carcinogenesis. miRNA expression is dysregulated in many types of human cancers, including thyroid cancer (8–11). In fact, most studies have demonstrated an alteration of miRNA expression in tissues derived from primary PTC compared with normal thyroid. Among all analyzed cases of PTC, miR-221, miR-222, and miR-181b have been found to be up-regulated, in some cases by more than 10-fold (12–14). In addition, miR-221 and miR-222 were more highly expressed in PTC than in benign thyroid nodules from formalin-fixed, paraffin-embedded tissue (13). Although tissue miRNA profiles may be useful for differentiating benign and malignant thyroid nodules, as mentioned, attainment of tissue requires an invasive procedure.

In contrast, blood sampling is minimally invasive and easy to obtain, making it attractive to explore for potential biomarkers. Recent studies have measured circulating miRNA, and the results suggest that serum miRNA profiles may be useful as cancer markers. A 2008 study investigating the sera of 60 patients with diffuse large B cell lymphoma and 40 controls reported that miRNA-21 expression was significantly higher in diffuse large B cell lymphoma patients (15). Other studies have shown the potential of circulating miRNA as minimally invasive markers for lung, colorectal, and breast cancers (16–18). To date, there are no reports on the possible utility of circulating miRNA quantification in patients with thyroid nodules.

In the present study, serum miRNA were measured by Solexa sequencing technology followed by extensive quantitative RT-PCR (qRT-PCR) validation. We compared the serum miRNA profiles of patients with PTC or benign thyroid nodules and healthy controls. In addition, serum miRNA profiles and tissue miRNA profiles were compared in patients with different clinicopathological features of PTC.

### Subjects and Methods

#### Participants

A total of 106 patients with primary PTC, 95 patients with benign thyroid nodules, and 44 healthy controls were enrolled. All patients were recruited at the First Affiliated Hospital and the Cancer Center of Sun Yat-sen University (Guangzhou, China). The 44 control subjects were age- and gender-matched healthy volunteers with no current or previous malignancy and no thyroid disease. The surgical procedure was performed on all patients, and final diagnoses were based upon pathological examination. The relevant demographic and clinicopathological information of patients with histologically confirmed PTC or benign nodules was recorded (Table 1). Tissue miRNA analysis was performed in subjects whose formalin-fixed, paraffin-embedded surgical samples were available (53 of 106 PTC patients and 53 of 95 benign nodule patients). In addition, repeat sera were collected from a subset of PTC patients after tumor resection (n = 9). All subjects provided informed consent, and the study was approved by the ethics committee of Sun Yat-sen University.

#### Sample processing and RNA/DNA extraction

miRNA from serum was isolated using the microRNA Fast Extraction Kit (Bioteke, Beijing, China) with minor modifications that are described in detail in Supplemental Text 1 (published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org).

#### Solexa sequencing and qRT-PCR analysis of miRNA

For initial biomarker screening, the sequencing procedure was conducted by Solexa sequencing as described in detail in Supplemental Text 1. Quantification of mature miRNA from 250 μl serum was performed as previously described (17). Real-time PCR was performed in duplicate using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The miRNA-specific primer sequences were designed based on the miRBase database (Supplemental Table 1). At present, no widely accepted endogenous control miRNA are available for study of circulating miRNA levels. miR-16 was used as a reference for serum miRNA analysis in several studies (15, 17, 18). Our Solexa sequencing results indicated that serum miR-16 levels were similar among the three sample groups (Table 2), so we used it as the endogenous serum control. The RNU6B was used as the reference for tissue expression of miRNA. Relative expression quantification of each miRNA was performed by the comparative cycle threshold (CT) method (2^(-ΔΔCT)) (19). In the serum study, expression values of target miRNA were calibrated relative to a pooled serum from 10 healthy controls. In the tissue study, expression values of target miRNA were calibrated to a pool of 10 benign nodule tissues. Fold change in expression of each miRNA was determined by the mean 2^-ΔΔCT value of the PTC group relative to the mean 2^-ΔΔCT value of the benign nodule group or healthy control group.
TABLE 1. Clinicopathological features and their correlation with serum levels of miRNA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients</th>
<th>let-7e</th>
<th>miR-151-5p</th>
<th>miR-222</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with PTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>81</td>
<td>2.50 ± 2.01</td>
<td>1.38 ± 0.97</td>
<td>2.61 ± 1.61</td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>1.96 ± 1.30</td>
<td>1.37 ± 1.09</td>
<td>2.79 ± 2.03</td>
</tr>
<tr>
<td>Age &lt;45 yr</td>
<td>65</td>
<td>2.52 ± 1.73</td>
<td>1.39 ± 0.96</td>
<td>2.60 ± 1.49</td>
</tr>
<tr>
<td>≥45 yr</td>
<td>41</td>
<td>2.13 ± 2.09</td>
<td>1.35 ± 1.06</td>
<td>2.74 ± 2.02</td>
</tr>
<tr>
<td>Tumor size ≤2 cm</td>
<td>67</td>
<td>2.25 ± 1.73</td>
<td>1.14 ± 0.60a</td>
<td>2.46 ± 1.59</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>39</td>
<td>2.58 ± 2.11</td>
<td>1.78 ± 1.35a</td>
<td>2.99 ± 1.87</td>
</tr>
<tr>
<td>Multifocal cancer</td>
<td>Yes</td>
<td>36</td>
<td>3.07 ± 2.25a</td>
<td>1.41 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>70</td>
<td>2.01 ± 1.56a</td>
<td>1.36 ± 1.08</td>
</tr>
<tr>
<td>Metastatic lymph node</td>
<td>Yes</td>
<td>60</td>
<td>2.68 ± 1.91</td>
<td>1.59 ± 1.09a</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>46</td>
<td>1.97 ± 1.78</td>
<td>1.10 ± 0.77a</td>
</tr>
<tr>
<td>TNM stage</td>
<td>IV</td>
<td>86</td>
<td>2.26 ± 1.76</td>
<td>1.32 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>III/V</td>
<td>20</td>
<td>2.85 ± 2.32</td>
<td>1.59 ± 1.17</td>
</tr>
<tr>
<td>AMES</td>
<td>Low risk</td>
<td>95</td>
<td>2.39 ± 1.92</td>
<td>1.34 ± 0.93</td>
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<tr>
<td></td>
<td>High risk</td>
<td>11</td>
<td>2.21 ± 1.58</td>
<td>1.71 ± 1.45</td>
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<tr>
<td>BRAF mutation</td>
<td>Yes</td>
<td>19</td>
<td>2.46 ± 1.52</td>
<td>1.77 ± 1.44</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>34</td>
<td>2.35 ± 1.93</td>
<td>1.43 ± 1.12</td>
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<tr>
<td>Tg ≤90.7 pmol/liter</td>
<td>69</td>
<td>2.43 ± 1.83</td>
<td>1.31 ± 0.95</td>
<td>2.54 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>&gt;90.7 pmol/liter</td>
<td>37</td>
<td>2.26 ± 1.98</td>
<td>1.51 ± 1.08</td>
</tr>
<tr>
<td>Patients with benign thyroid nodule</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>71</td>
<td>0.89 ± 0.36</td>
<td>0.64 ± 0.42</td>
<td>0.76 ± 0.54</td>
</tr>
<tr>
<td>Male</td>
<td>24</td>
<td>1.04 ± 0.45</td>
<td>0.63 ± 0.31</td>
<td>0.91 ± 0.52</td>
</tr>
<tr>
<td>Age &lt;45 yr</td>
<td>60</td>
<td>0.96 ± 0.39</td>
<td>0.64 ± 0.41</td>
<td>0.83 ± 0.58</td>
</tr>
<tr>
<td>≥45 yr</td>
<td>35</td>
<td>0.87 ± 0.39</td>
<td>0.63 ± 0.37</td>
<td>0.76 ± 0.46</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>32</td>
<td>0.94 ± 0.97</td>
<td>0.67 ± 0.62</td>
<td>0.80 ± 0.87</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>1.04 ± 0.93</td>
<td>0.59 ± 0.60</td>
<td>0.71 ± 0.66</td>
</tr>
<tr>
<td>Age &lt;45 yr</td>
<td>26</td>
<td>0.88 ± 0.71</td>
<td>0.65 ± 0.60</td>
<td>0.80 ± 0.81</td>
</tr>
<tr>
<td>≥45 yr</td>
<td>18</td>
<td>1.06 ± 1.30</td>
<td>0.68 ± 0.67</td>
<td>0.79 ± 0.89</td>
</tr>
</tbody>
</table>

TNM stage was assessed according to International Union Against Cancer (UICC) staging systems; AMES is a risk definition including age, metastases, extent, and size. Mean ± SD is presented as the mean 2−ΔΔCT ± SD of target miRNA expression values.

a P < 0.05 (P value was determined by one-way ANOVA).

**BRAF mutation**

Mutations in the BRAF gene were identified by direct DNA sequencing. For sequencing, exon 15, which contains the locus of the T1796A mutation, was amplified using primer pairs (Supplemental Table 1), as previously reported (20).

**Statistical analysis**

All statistical analyses were performed using SPSS software (version 16.0). The Mann-Whitney U test and the paired t test were used to determine the significance of different levels of miRNA expression. Receiver operating characteristic (ROC) curves were used to analyze the diagnostic utility of differentially expressed miRNA. A logistic regression model determined the predicted probability of the combination of three miRNA. The optimal cutoff point was chosen as the value at which the sum of sensitivity and specificity was maximal. The levels of miRNA in each group were defined as mean ± SD. The Pearson correlation coefficient was used to compare the miRNA levels in sera and tissues. One-way ANOVA was used to identify possible associations between miRNA concentrations and clinicopathological features of PTC patients. All P values were two-sided, and a P value < 0.05 was considered statistically significant.

**Results**

**Serum miRNA in PTC, benign nodule, and healthy control groups**

We used Solexa sequencing to measure the expression of serum miRNA in patients with PTC or benign thyroid
nODULES AND HEALTHY CONTROLS. OUR RESULTS IDENTIFIED 372
miRNA IN THE PTC GROUP, 321 IN THE BENIGN NODULE GROUP,
AND 375 IN THE HEALTHY CONTROL GROUP (SUPPLEMENTAL TABLE
2). AMONG THE 372 SERUM miRNA IN THE PTC GROUP, 274
WERE PRESENT IN ALL THREE GROUPS AND 34 WERE PRESENT ONLY
IN THE PTC GROUP (FIG. 1A). THE OVERALL EXPRESSION LEVELS
OF INDIVIDUAL SERUM miRNA IN THE PTC GROUP WERE SIMILAR
TO THAT IN THE BENIGN NODULE GROUP (FIG. 1B) AND HEALTHY
CONTROL GROUP (FIG. 1C). THESE RESULTS INDICATE THAT MOST OF
THE miRNA EXPRESSION LEVELS ARE SIMILAR IN THESE THREE
GROUPS BUT THAT SOME SERUM miRNA MAY BE USEFUL AS
MARKERS OF PTC.

Next, we searched for potential miRNA signatures to
distinguish PTC patients from those with benign nodules
and healthy controls. Our results identified 372
miRNA in the PTC group, 321 in the benign nodule group,
and 375 in the healthy control group (Supplemental Table
2). Among the 372 serum miRNA in the PTC group, 274
were present in all three groups and 34 were present only
in the PTC group (Fig. 1A). The overall expression levels
of individual serum miRNA in the PTC group were similar
to that in the benign nodule group (Fig. 1B) and healthy
control group (Fig. 1C). These results indicate that most of
the miRNA expression levels are similar in these three
groups but that some serum miRNA may be useful as
markers of PTC.

Next, we searched for potential miRNA signatures to
distinguish PTC patients from those with benign nodules
and healthy controls. miRNA were selected for
qRT-PCR validation studies based on three criteria: 1)
at least 30 copies in the PTC, benign nodule, and healthy
control groups, 2) at least 5-fold change in expression in
the PTC group compared with the benign nodule group
and healthy control group, and 3) up-regulation in the
PTC group. Analysis of down-regulated miRNA would
have resulted in poor sensitivity and specificity because of
the very low serum levels of miRNA. Based on these
criteria, we identified seven miRNA (miR-181b, miR-1975,
miR-144, miR-100, miR-151-5p, miR-222, and miR-
543) that differentiate the PTC group from the benign
nodule group and five miRNA (miR-100, miR-151-5p,
miR-222, miR-543, and miR-127-3p) that differentiate
the PTC group from the healthy control group (Table 2).
Serum miR-100, miR-151-5p, miR-222, and miR-543
satisfied all of the above criteria; thus, these four miRNA
were analyzed by qRT-PCR. We also found that let-7e was
elevated by more than 3-fold in the PTC group. Previous
research indicated that members of the let-7 miRNA fam-
ily act as tumor suppressors in many cancers from solid
organs (21). Therefore, it was also included as a potential
serum marker of PTC.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Raw copy number</th>
<th>Fold change&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7e</td>
<td>23682</td>
<td>3.07</td>
</tr>
<tr>
<td>miR-181b</td>
<td>331</td>
<td>5.51</td>
</tr>
<tr>
<td>miR-1975</td>
<td>846</td>
<td>5.89</td>
</tr>
<tr>
<td>miR-144</td>
<td>1749</td>
<td>6.13</td>
</tr>
<tr>
<td>miR-100</td>
<td>965</td>
<td>5.35</td>
</tr>
<tr>
<td>miR-151-5p</td>
<td>345</td>
<td>5.44</td>
</tr>
<tr>
<td>miR-222</td>
<td>37043</td>
<td>5.82</td>
</tr>
<tr>
<td>miR-543</td>
<td>316</td>
<td>5.41</td>
</tr>
<tr>
<td>miR-127-3p</td>
<td>227</td>
<td>3.78</td>
</tr>
<tr>
<td>miR-16</td>
<td>45528</td>
<td>0.75</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold change was calculated using normalized miRNA expression. Normalized miRNA expression = (raw copy number/total clean reads count of each group) × 1,000,000. Total clean reads count refers to the small RNA reads, filtrating reads contaminated by rRNA, tRNA, mRNA, small nuclear RNA, and small nucleolar RNA.

FIG. 1. Expression of serum miRNA in the PTC group, benign thyroid nodule group, and healthy control group. A, Set diagram showing miRNA expression in patients with PTC or benign thyroid nodules and healthy controls; B, scatter plot of expression of specific miRNA in the PTC and the benign nodule groups; C, scatter plot of expression of specific miRNA in the PTC and the healthy control groups. In B and C, each point represents a specific miRNA; red points represent miRNA that were up-regulated at least 2-fold, blue points represent miRNA whose expression was 0.5- to 2-fold that of the control (equally expressed), and green points represent miRNA that were down-regulated at least 2-fold. The expression level represents normalized miRNA copy number.
Expression profiles of five serum miRNA

We used qRT-PCR to measure the expression of serum let-7e, miR-100, miR-151-5p, miR-222, and miR-543 from all 245 enrolled patients, including the 30 patients used in the Solexa sequencing study (Fig. 2). The results indicated that serum levels of let-7e, miR-151-5p, and miR-222 were significantly higher in PTC patients than in patients with benign nodules and healthy controls (P < 0.05 for all comparisons). The levels of serum miR-100 (B) and miR-543 (E) were not significantly different in the PTC group relative to the benign nodule group and the healthy control group (P > 0.05 for all comparisons). For all five miRNA, there was no significant difference in expression between the benign nodule group and the healthy control group (P > 0.05 for all comparisons). The relative expression of the miRNA is presented as the log10 of target miRNA expression values. The lines inside the boxes denote the medians. All indicated P values were determined by the Mann-Whitney U test.

Predictive value of miRNA

To evaluate the diagnostic value of serum let-7e, miR-151-5p, and miR-222 for PTC, ROC curve analysis was performed. A comparison of the PTC and benign nodule groups indicated that use of all three miRNA had an area under the ROC curve (AUC) of 0.917 [95% confidence interval (CI) = 0.878–0.955], with 87.8% sensitivity and 88.4% specificity at the cutoff value of 0.41 (Fig. 3A). Individually, let-7e had an AUC of 0.782 (95% CI = 0.716–0.848), miR-151-5p an AUC of 0.780 (95% CI = 0.717–0.844), and miR-222 an AUC of 0.890 (95% CI = 0.866–0.945). At the cutoff values of 1.41 for let-7e, 1.08 for miR-151-5p, and 1.39 for miR-222, sensitivity and specificity were 63.2 and 89.5, 59.4 and 89.5, and 81.1 and 89.5%, respectively (Supplemental Fig. 2A).

Comparison of the PTC group and the healthy control group indicated that use of all three miRNA had an AUC of 0.897 (95% CI = 0.839–0.955), with 86.8% sensitivity and 79.5% specificity at the cutoff value of 0.58 (Fig. 3B). Individually, let-7e had an AUC of 0.786 (95% CI = 0.708–0.865), miR-151-5p an AUC of 0.780 (95% CI = 0.717–0.844), and miR-222 an AUC of 0.882 (95% CI = 0.817–0.947). At the cutoff values of 0.99 for let-7e, 0.68 for miR-151-5p, and 0.80 for miR-222, sensitivity and specificity were 63.2 and 89.5, 59.4 and 89.5, and 81.1 and 89.5%, respectively (Supplemental Fig. 2B). ROC curve analysis indicated that these three miRNA were unable to distinguish patients with benign nodules from healthy subjects (data not shown). Considering the potential of serum let-7e, miR-151-5p, and miR-222 as markers for PTC, we measured the levels...
of these miRNA in a subset of PTC patients (n = 9) before and 5–15 d after tumor excision. The serum levels of miR-151-5p and miR-222 decreased significantly after surgery in all nine patients (P < 0.001 and P < 0.005, respectively) (Fig. 3, D–E). The serum levels of let-7e were also decreased in seven of nine patients after surgery (P < 0.081) (Fig. 3C). The postoperative serum levels of miR-151-5p and miR-222 in PTC patients were comparable to the levels in healthy subjects (P = 0.056 and P = 0.169, respectively).

Correlation of miRNA expression in tissue and serum

Giving that levels of let-7e, miR-151-5p, and miR-222 were significantly higher in the serum of PTC patients than in those with benign nodules and healthy subjects, we proceeded to use a qRT-PCR assay to screen for expression of these three miRNA in a cohort of PTC and benign nodule tissues. There was a significant correlation between the tissue and serum expression of miR-151-5p and miR-222 in patients with PTC (Pearson correlation coefficient R = 0.33, P = 0.016; and R = 0.588, P < 0.001, respectively), however, no significant correlation for let-7e expression (R = −0.108, P = 0.442) was found (Fig. 4, A–C). There was no significant difference in the expression of let-7e in PTC tissues and benign nodule tissues (0.907 vs. 0.724, P = 0.349). However, the median levels of tissue miR-151-5p and miR-222 were significantly higher in patients with PTC than in those with benign nodules (1.726 vs. 0.734, P < 0.001; and 1.417 vs. 0.182, P < 0.001, respectively) (Fig. 4, D and E).

Correlation of miRNA and clinicopathological features of PTC

Observing that circulating miRNA (let-7e, miR-151-5p, and miR-222) and tissue miRNA (miR-151-5p and miR-222) were significantly elevated in PTC patients, we assessed the relationship between systemic miRNA profiles and clinicopathological features (Table 1). The results indicated that the levels of serum miR-151-5p and miR-222 were significantly higher in lymph node-positive patients than in lymph node-negative patients (P = 0.012 and P = 0.001, respectively). Our results also indicated that overexpression of serum miR-151-5p was strongly associated with tumor size (P < 0.001) and that overexpression of serum miR-222 was associated with advanced Tumor-Node-Metastasis (TNM) stage (P = 0.015). In addition, patients with multifocal lesions had significantly higher levels of serum let-7e than those with unifocal lesions (P < 0.001).

We also examined the correlation of clinicopathological features and miRNA expression in paired tissue and serum samples (Supplemental Fig. 3). Patients with positive lymph nodes and larger tumors had increased expression of miR-151-5p (tissue: P = 0.015 and P = 0.046; serum: P = 0.034 and P < 0.001, respectively). Patients with positive lymph nodes and advanced TNM stage had higher expression of miR-222 (tissue: P = 0.042 and P =
Possible relationships between miRNA levels and sex, age, BRAF mutation, and serum thyroglobulin (Tg) were also analyzed, but no significant correlations were observed for any of these variables (Table 1).

Discussion

The current approaches for differential diagnosis of benign nodules from malignancy include history, physical examination, ultrasound, thyroid nuclear scanning, FNA, and surgical pathological examination. The gold standard is surgical pathological examination, and FNA is considered the most reliable method for preoperative diagnosis of thyroid nodules. However, predictive value of FNA is still limited in subjects with cytological features of suspicious malignancy or follicular neoplasm and atypia of undetermined significance, leading to unnecessary thyroid lobectomies or thyroidectomy (7).

Previous studies have reported that certain circulating miRNA have potential utility as diagnostic or prognostic markers for numerous cancers (22). Given their reproducible and stable existence in sera, miRNA profiles have emerged as a way to classify a wide variety of human cancers (16–18). Most previous studies examined miRNA expression mainly in tumor cells and tissues from PTC patients. However, no studies have examined circulating miRNA expression in PTC patients, and none have attempted to use serum miRNA to differentiate benign nodules from malignancies.

In the present study, we performed initial screening using Solexa sequencing to measure serum miRNA expression followed by extensive qRT-PCR validation in patients with PTC or benign nodules and in healthy subjects. We identified a profile in which three miRNA (let-7e, miR-151-5p, and miR-222) were up-regulated in the sera of PTC patients compared with patients with benign nodules and healthy controls. However, no studies have examined circulating miRNA expression in PTC patients, and none have attempted to use serum miRNA to differentiate benign nodules from malignancies.
genes, including 27) because it inhibits the expression of multiple onco-
fingerprint. The serum miRNA profile serves as a tumor finding that tumor cells are the source of serum miRNA, which may be released from tumor cells or a product of tumor cell death and lysis. The finding that tumor cells are the source of serum miRNA supports that serum miRNA profile serves as a tumor fingerprint.

The let-7 family is considered a tumor suppressor (26, 27) because it inhibits the expression of multiple oncogenes, including RAS, MYC, and HMGA2. In the present study, we found discrepant expression of let-7e in tissue and sera. Similarly, Heneghan et al. (18) reported that expression of let-7a was greatly increased in the blood of breast cancer patients. Our results indicate that serum let-7e, a tumor-related miRNA, might be a potential marker for PTC, but the relationship of serum let-7e with tumorigenesis deserves further study.

Our analysis indicated that elevated serum miR-222 was associated with lymph node metastasis and advanced TNM stage in PTC patients, whereas miR-151-5p was associated with lymph node metastasis and larger tumor size. Previous reports indicated that miR-222 was up-regulated in tissue specimens of PTC and that this miRNA, which targets p27, p57, and PUMA, may play an essential role in thyroid oncogenesis (28–30). Ding et al. (31) reported that miR-151 significantly increased hepatocellular carcinoma cell migration and invasion in vitro and in vivo, mainly through miR-151-5p but not miR-151-3p. miR-151-5p resides within intron 22 of the host gene encoding focal adhesion kinase (FAK), and its expression highly correlated with FAK expression (31). FAK, over-expressed in numerous human tumors including PTC, is a nonreceptor tyrosine kinase that acts as a primary regulator of focal adhesion signaling, which regulates cell invasion and metastasis (32). We are currently investigating the correlation of FAK and miR-151-5p expression in tissues and cell lines of thyroid carcinoma, which might provide us new insights into the mechanism of invasion and metastasis of PTC.

We analyzed the association of up-regulated serum miRNA with serum Tg levels, which are elevated in most patients with relapsed PTC and follicular thyroid carcinoma and thus are used as a tumor marker for recurrence but not as a tool for initial evaluation of thyroid nodules (33). No correlation was found between serum miRNA and Tg levels. We also found no significant correlation between serum miRNA levels and tumor BRAFV600E mutation, one of the most prevalent mutations in PTC (34). Our finding suggests that altered expression of miRNA might occur independently from BRAFV600E mutation, and other epigenetic mechanisms might be responsible for their dysregulation.

The dynamic expression patterns of circulating miRNA have not yet been clearly elucidated. In the present study, we found that expression of serum miR-151-5p and miR-222 decreased significantly after surgical resection of primary PTC; however, let-7e did not decrease significantly compared with preoperative levels. The limitation of this study is the small number of patients examined. Given the alteration of these miRNA levels after surgical removal of tumors, studies with larger patient samples are needed to evaluate the possibility of these miRNA as markers for monitoring tumor recurrence and predicting prognosis.

In conclusion, our study showed that certain serum miRNA are up-regulated in PTC. The circulating miRNA let-7e, miR-151-5p, and miR-222 may serve as novel minimally invasive biomarkers for the diagnosis of PTC. These findings provide us with a foundation for the development of an easy, minimally invasive, and effective diagnostic tool for preoperative assessment of thyroid nodules. Long-term follow-up of the patients in the current study and a prospective study with a larger sample size are needed to further validate the usefulness of circulating miRNA in the diagnosis of thyroid carcinoma.

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Address all correspondence and requests for reprints to: Haipeng Xiao, Department of Endocrinology, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan Road 2, Guangzhou, China 510080. E-mail: xiaohp@mail.sysu.edu.cn.

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References


5. Gibas ES, Ali SZ 2009 The Bethesda System for Reporting Thyroid Cytopathology. Thyroid 19:1159–1165


