MicroRNA analysis as a potential diagnostic tool for papillary thyroid carcinoma

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MicroRNA (miRNA) microarray analysis has consistently found altered expression of miRNAs in thyroid tumors, suggesting their roles in thyroid carcinogenesis. To explore whether this differential expression can be used as a diagnostic tool in surgical pathology and fine-needle aspirate (FNA) specimens, the expression of selected miRNA was evaluated by quantitative RT-PCR, using total RNA from 84 formalin-fixed paraffin-embedded tissues and 40 *ex vivo* aspirate specimens. miRNA from all paraffin-embedded tissues and all but one FNA sample were found to be analyzable, with paraffin sections yielding better miRNA quality. Preliminary analysis of 6 miRNAs in 10 papillary thyroid carcinoma and 10 follicular adenoma identified significant overexpression of miR-146b, -221, and -222 in papillary thyroid carcinoma ($P < 0.02$), but not miR-146a, -155, or -187 ($P > 0.08$). The expression of these first three miRNAs was examined in a series of 5 normal thyroid, 11 hyperplastic nodules, 24 follicular adenoma, 27 classical papillary thyroid carcinoma, 5 follicular variant papillary thyroid carcinoma, 2 follicular carcinoma, and 10 encapsulated follicular lesions with partial nuclear features of papillary carcinoma. Results showed miR-146b to be most consistently overexpressed in both classical papillary carcinoma and follicular variants, whereas all other groups showed lower expression at a similar level ($P < 0.001$ for pair-wise comparisons between papillary carcinoma and all other groups). Follicular lesions with partial features of papillary carcinoma all showed low miR-146b levels similar to other non-papillary carcinoma groups, suggesting that they are biologically distinctive from papillary carcinoma. miR-221 and miR-222 also showed higher expression in papillary carcinoma, but with substantial overlaps with the other groups. When applied to 40 FNA samples of various lesions, only miR-146b and miR-222 persisted as distinguishing markers for papillary carcinoma. We concluded that miRNAs, particularly miR-146b, might potentially be adjunct markers for diagnosing papillary thyroid carcinoma in both FNA and surgical pathology specimens.

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MicroRNAs (miRNAs) represent a class of small RNA that has been identified only recently. First described in *Caenorhabditis elegans* in 1993,¹ miRNAs were subsequently found to be conserved in various plant and animal species in 2000.² Unlike mRNA, miRNAs are only 19–25 nucleotides in size and do not encode amino-acid sequences. In contrast to the vast diversity of mRNA transcripts, the number of miRNA species is much smaller, with 851 human miRNAs reported to date (December 2007, miRBase, Sanger Institute). Despite this limited complexity, miRNAs have been shown to be of

profound biological importance, negatively regulating gene expression at the post-transcriptional level. It is estimated based on sequence complementarity that each miRNA can potentially bind to hundreds of different mRNA species. Such binding, which occurs mostly at the 3'-untranslated regions of mRNA transcripts but may also occur at the 5'-untranslated or the coding region, could lead to decreased protein expression of the target gene, either by follicular adenomacilitating degradation of the target mRNA or by suppression of the translational machinery. Through these negative regulatory mechanisms, miRNA have been shown to affect various biological processes in both normal and diseased states, including tumorigenesis in human (for reviews, see references Croce and Calin; Calin and Croce; Zhang W *et al*^{3–5}).

By using array-based technology and reverse transcription (RT)-PCR, the global miRNA expression

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profiles as well as the level of individual miRNA species have been analyzed in various human cancers, and aberrant miRNA expression was found in all tumor types studied to date, including hematological malignancies,⁶ lymphoma,⁷ melanoma,⁸ glioblastoma,^{9,10} as well as epithelial cancers from lung,^{11,12} breast,^{13,14} colon,^{15,16} liver,^{17,18} thyroid,^{19–22} pancreas^{23–26} etc. When compared to their normal-tissue counterparts, both overexpression and underexpression of miRNA has been observed, with decreased expression appearing to be more common in some studies,^{8,27} leading to the hypothesis that oncogenes might be target genes for these miRNAs. These observed changes in miRNA also implied that miRNA could potentially be useful diagnostic and prognostic markers in cancer, and may even be therapeutic targets. This cancer biology aspect of miRNA has been the subject of multiple recent reviews.^{3,5,28–38}

In thyroid tumors, miRNA changes have been found in papillary thyroid carcinoma,^{21,22,39} follicular carcinoma,¹⁹ and anaplastic carcinoma.²⁰ In anaplastic carcinoma, the main changes appeared to be underexpression, and a significant decrease of miR-30d, miR-125b, miR-26a, and miR-30a-5p expression was described in comparison to normal thyroid tissue. In contrast, overexpression of a selected number of miRNA over normal thyroid was found in follicular carcinoma and in papillary carcinoma. Four miRNAs (miR-192, miR-197, miR-328, and miR-346) were found to show increased expression in follicular carcinoma, whereas a non-overlapping group of miRNAs was found to have increased expression in papillary carcinoma, including miR-221, miR-222, miR-146,^{21,22,40} miR-181b,²² miR-155, miR-187⁴⁰ etc.

In this study, we sought to further validate the overexpression of several selected miRNA species in papillary thyroid carcinoma—both classical type and follicular variants—and evaluate their potential diagnostic value by comparing the miRNA levels to those seen in other follicular lesions, particularly the commonly encountered follicular adenoma and hyperplastic nodules. Both formalin-fixed paraffin-embedded surgical resection material and fresh-frozen *ex vivo* fine-needle aspirate (FNA) material were evaluated and compared to seek the potential utility of miRNA in these two settings.

Materials and methods

Case Selection and Tumor Tissue Procurement

Surgical pathology database from Department of Pathology, Weill Cornell Medical College was searched from 2003 to present for thyroidectomy specimens and cases diagnosed as papillary thyroid carcinoma, follicular adenoma, hyperplastic nodules, and follicular carcinoma were identified. The H&E slides from selected cases were reviewed by two of us (TS and Y-TC) and only unequivocal

cases were used. Follicular lesions with partial nuclear features of papillary carcinoma were identified and evaluated separately. A representative block was chosen from each case, and two to four 10- μ m unstained sections were used for RNA extraction. The nontumor areas on the paraffin slides were manually removed with surgical blades, and the remaining tissue on the slide was scraped into an Eppendorf tube for RNA extraction. For normal thyroid, uninvolved thyroid tissue from the follicular adenoma cases was used, taken from tissue blocks that were histologically unremarkable thyroid tissue.

FNA specimens were obtained from thyroidectomy specimens performed by one of the authors (TJF) from 2002 to 2005 at the New York Presbyterian Hospital, Weill Cornell Medical Center. FNAs of the nodules were performed on the *ex vivo* specimens with five passes of a 23-gauge needle with 10 ml syringe and the aspirated material was suspended in RLT lysis buffer (Qiagen Inc., Valencia, CA, USA), snapped frozen in liquid nitrogen and stored at -80°C until processing. Diagnoses on these FNA cases were based on final surgical pathology report and confirmed by slide review. All tissues were obtained with the informed consent of each patient and in accordance with approved protocols and guidelines of our internal review board.

RNA Extraction

RNA from paraffin-embedded tissues was extracted using Ambion RecoverAll Total Nucleic Acid Isolation kit. For FNA samples, the initial lysis protocol was modified as these samples were initially collected in the RLT lysis buffer of the Qiagen RNeasy kit. For these specimens, 200–400 μ l of lysis buffer from the Ambion mirVana miRNA isolation kit was added, and total RNA was then extracted organically using acid phenol:chloroform and was purified through glass-fiber-filtered column, following manufacturer's protocol. The quantity of total RNA isolated was from 1.1 to 27 μ g for the paraffin-embedded samples and 0.41–12 μ g for the FNA specimens.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed using an ABI PRISM 7000 Sequence Detection System. Reverse transcription for each specific miRNA was performed using 10 ng RNA for 10 μ l reverse transcription reaction, and 1 μ l cDNA was then used for each 20 μ l PCR (TaqMan MicroRNA Assay kit, Eurogentec qPCR mastermix; Applied Biosystems). Total 45 cycles of amplification were performed, each cycle consisting of 15 s at 95°C and 1 min at 60°C . Control amplification for endogenous small RNA U6 was performed in all samples.

Following the amplification, the same threshold was set for analyzing all experiments to compare C_t values derived from different experiments. Mean C_t values were calculated for each specimen and then normalized against the corresponding U6 C_t values, calculated as $(C_{t(\text{Experimental gene}} - C_{t(\text{U6})})$. All data presented were normalized C_t values.

Statistical Analysis

In the first part of the experiments, Student's *t*-tests were used to compare the expressions of each of the six candidate miRNAs between papillary thyroid carcinoma and follicular adenoma (10 cases each). Markers that exhibited significant difference (Bonferroni adjusted *P*-value < 0.05) between the two groups were further analyzed in expanded lists of fresh and formalin-fixed tissue samples. Tukey's test was used to compare the expression values of a marker across multiple groups pairwise, although properly adjusting for multiple comparisons. Pearson's correlation coefficient was used to quantify the correlation between the expression values of any two markers. All statistical analyses were carried out using Open Source Statistical software package R (www.r-project.org).

Results

Selection of miRNA

Six miRNA (miR-146a, miR-146b, miR-155, miR-187, miR-221, and miR-222) were selected for initial testing based on the literature.^{21,22,40} Ten cases each of papillary thyroid carcinoma (eight classical and two follicular variant) and follicular adenoma were analyzed using paraffin-embedded materials. All cases showed uniformly high miRNA quality, with C_t values for control U6 RNA RT-PCR clustering within <3 amplification cycles for all samples (Figure 1a). Significant overexpression of miR-146b, miR-222, and miR-221 was found in papillary carcinoma compared to follicular adenoma, with Bonferroni adjusted *P*-values being < 0.0001, 0.0028, and 0.014, respectively. In contrast, no significant difference was seen for miR-146a, miR-155, or miR-187 (*P* = 0.08, 0.10, and 0.64, respectively).

miRNA Differential Expression in Paraffin-Embedded Tissues

The expression analysis of miR-146b, miR-221, and miR-222 was expanded to a series of 74 specimens, including 32 papillary thyroid carcinoma (27 classical and 5 follicular variant), 24 follicular adenoma, 11 hyperplastic nodules, 2 follicular carcinoma, and 5 normal thyroid tissues. These four later groups will be collectively referred to as 'non-papillary carcinoma groups'. The normalized C_t distribution

is shown in Figure 2. miR-146b showed the best separation between the papillary carcinoma and non-papillary carcinoma groups (*P* < 10⁻¹⁸ and *P* < 0.001 for all pair-wise comparison between papillary carcinoma and individual groups), with 28 of the 32 papillary carcinoma having miR-146b expression levels higher than the 42 non-papillary carcinoma cases. No difference was found between classical papillary carcinoma group and the follicular variant group (*P* = 0.81). By setting a cutoff normalized C_t value at -0.285 (papillary thyroid carcinoma ≤ -0.285, non-papillary carcinoma groups > -0.285), this classification scheme would have a sensitivity of 93.8% and a specificity of 97.6%. All non-papillary carcinoma groups, ie follicular adenoma, hyperplastic nodules, normal thyroid, and the two cases of follicular carcinoma, showed lower levels of miR-146b expression and were indistinguishable from each other (*P*-values ranged from 0.79 to 0.99 for all pair-wise comparisons).

For miR-221 and miR-222, significantly higher expression was similarly seen in papillary thyroid carcinoma than in follicular adenoma, hyperplastic nodules, and normal thyroid (*P* < 0.001 for all pair-wise comparisons and for papillary thyroid carcinoma vs non-papillary carcinoma groups for both miRNAs). However, as shown in Figure 2, substantial overlaps were seen between the miRNA distribution ranges among the various groups, and for individual cases it would be impossible to distinguish papillary thyroid carcinoma from other lesions based on the expression of miR-221 and/or miR-222. Also different from miR-146b was the finding that the two cases of follicular carcinoma showed intermediate levels of miR-221 and miR-222 expression, not separable from papillary thyroid carcinoma or other groups.

Similar to previously described,²² strong correlation was seen between expression of miR-221 and miR-222 (*r* = 0.963). In comparison, weaker (but still moderate to strong) correlations were seen between the expression of miR-146b and miR-221 and miR-222 (*r* = 0.795 and 0.835, respectively).

miRNA in 'Borderline' Follicular Lesions

Ten cases of encapsulated follicular lesions with partial nuclear features (nuclear clearing and occasional grooves) were previously identified and characterized in a preliminary report.⁴¹ These cases showed immunohistochemical phenotypes (HBME1, Galectin3, CK19 staining characteristics) and mRNA expression profiles (by DNA microarray) intermediate of follicular adenoma and papillary carcinoma, leading us to conclude that these likely represent 'borderline' lesions between follicular adenoma and follicular variant of papillary carcinoma. qRT-PCR showed all 10 cases to have low miR-146b expression levels, indistinguishable from

Figure 1 qRT-PCR amplification of endogenous small RNA U6, using total RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues (**a**) or from fresh-frozen *ex vivo* FNA samples (**b**, see text below). The x axis shows the cycle numbers and the y axis depicts the ΔRn . FFPE samples yielded better quality small RNA, evidenced by tighter threshold cycle number (C_t) distribution among the 20 samples analyzed than the similarly analyzed FNA samples.

follicular adenoma ($P=0.998$) and significantly lower from both classical and follicular variants of papillary carcinoma ($P<0.001$; Figure 2). In contrast, miR-221 and miR-222 showed expression patterns intermediate of papillary carcinoma (classical and follicular variants combined) and follicular adenoma but were not statistically different from either the adenoma group ($P=0.222$ and

0.115, respectively) or the papillary carcinoma group ($P=0.588$ and 0.381, respectively).

miRNA Analysis in Fine-Needle Aspirate Specimens

Forty *ex vivo* FNA specimens (10 each of hyperplastic nodules, follicular adenoma, classical

Figure 2 Histogram of the miR-146b, 221, and 222 expression in normal thyroid and in different thyroid lesions (normal, normal thyroid; HYP, hyperplastic nodule; FA, follicular adenoma; PTCFV, papillary thyroid carcinoma, follicular variant; PTCC, papillary thyroid carcinoma, classical type; FC, follicular carcinoma; FA/PTC, borderline lesions between follicular adenoma and papillary thyroid carcinoma, see text). The x axis depicts normalized C_t values, with higher numbers indicating higher expression of the microRNA species analyzed. The y axis depicts case number, with each bar representing an individual case. Overexpression of miR-146b was evident in almost all cases of classical and follicular variant papillary carcinoma, but not in non-papillary carcinoma specimens, including the 10 borderline lesions. miR-221 and miR-222 were similarly overexpressed in classical and follicular variants of papillary carcinoma (see text).

papillary thyroid carcinoma, and follicular variant papillary thyroid carcinoma) were then analyzed for the expression of miR-146b, miR-221, and miR-222. The RNA yields in this group (0.41–12 μ g) were lower than those in the paraffin-embedded group (1.1–27 μ g). The RNA quality was also poorer, evidenced by a broader range of A260/A280 ratios (1.61–2.82 vs 1.90–2.12) and more variable amplification of the endogenous control U6 small RNA (Figure 1b). Using the same input RNA quantity, the C_t range for U6 control RNA varied from 27.3 to 34.3 cycles for the FNA group, vs a much narrower range

of 25.5–28.4 cycles for the paraffin-embedded tissue group.

miRNA qRT-PCR results are summarized in Figure 3. Increased expression of all three miRNA was confirmed, but only miR-146b and miR-222 persisted as statistically significant distinguishing markers between the 20 papillary carcinomas (10 classical and 10 follicular variants) and the group of 20 benign lesions ($P=0.0002$ and 0.021 , respectively), whereas miR-221 did not ($P=0.105$). In pair-wise comparison treating all 20 papillary carcinoma cases as a group, miR-146b showed significantly higher expression in this group than in follicular adenoma ($P=0.003$) and hyperplastic nodules ($P=0.002$). In comparison, the expression levels of miR-221 and miR-222 were only significantly different between the papillary carcinoma group and follicular adenoma ($P=0.04$ and 0.005 , respectively), but not between papillary carcinoma and hyperplastic nodules ($P=0.596$ and 0.402 , respectively). No significant differences were seen in any other pair-wise comparisons for all three miRNA, including classical papillary thyroid carcinoma vs the follicular variants. Strong correlations were again seen between the expression of these three miRNA ($r>0.80$), strongest between miR-221 and miR-222 ($r=0.955$).

Discussion

Human miRNAs, identified within the past decade, have quickly emerged as potentially useful diagnostic and prognostic markers in cancer. In the present study, we showed that of several miRNAs previously demonstrated to be overexpressed in papillary thyroid carcinoma, miR-146b was most consistently altered, and that this might contribute to the differential diagnosis of this lesion from follicular adenoma and hyperplastic nodules in the future, potentially applicable to both formalin-fixed samples in surgical pathology and FNA samples. We and others have previously shown that papillary thyroid carcinoma could be separated from benign nodules, ie follicular adenomas and hyperplastic nodules, by their mRNA expression profiles using cDNA microarray analysis.^{42–44} By qRT-PCR evaluation, our preliminary data also showed that by using fresh-frozen materials, most cases of papillary carcinoma and follicular adenoma can also be separated by their expression of a small panel of several genes, namely *CK19*, *CITED1*, galectin 3, deiodinase 1, thyroglobulin, and pendrin.⁴⁵ However, these mRNA-based assays were found to be less reliable when applied to formalin-fixed, paraffin-embedded tissues, primarily due to the suboptimal RNA qualities in these specimens (unpublished data). In sharp contrast, we found in this study that miRNAs were uniformly well preserved in the formalin-fixed paraffin-embedded tissues, presumably due to their small size. This advantage indicates

Figure 3 Box-and-whisker plots of miR-146b, miR-221, and miR-222 expression in fine-needle aspirate samples derived from follicular adenoma (FA), hyperplastic nodules (HYP), follicular variant of papillary carcinoma (PTCFV), and classical papillary carcinoma (PTCC). Both classical and follicular variants of papillary carcinoma showed statistically higher expression of miR-146b than follicular adenoma and hyperplastic nodules; miR-221 and miR-222, although showing similar trends, were statistically less significant among the groups (see text for details).

that miRNA-based assay is most well suited for the molecular analysis of routinely processed formalin-fixed tissues, and additional research to construct an ideal differentially expressed miRNA panel and to validate such a panel by a larger scale analysis would be worthwhile. Along similar consideration, it is also significant to note that miR-146b was not overexpressed in the group of diagnostically difficult lesions that we tentatively termed 'borderline lesions' between follicular adenoma and follicular variant of papillary carcinoma. However, whether this lack of miR-146b expression can be used as evidence that these lesions are not full-blown papillary carcinomas would need to be proven by long-term follow-up studies on a larger number of such cases, and such studies are unfortunately difficult to carry out, given the very indolent clinical course of papillary carcinoma in general.

As to the potential diagnostic value of miRNA in the preoperative evaluation of thyroid nodules, our study using *ex vivo* FNA specimens showed overexpression of miR-146b in the papillary carcinoma group similar to that observed in the resection specimens, and miRNA yield was adequate in all but one case. However, the ranges of expression between papillary carcinoma and other groups overlapped more than in the paraffin-embedded tissues, limiting the diagnostic value at this setting. We suspect that this suboptimal result might partially be due to technical reasons, as the miRNA quality was variable among our FNA samples, and a subsequent modification in the initial specimen collection and cell lysis step appeared to have improved the miRNA quality (data not shown). Whether this would improve the diagnostic utility

of miRNA in preoperative *in vivo* FNA samples is being tested prospectively in a larger series of samples.

In addition to diagnostic considerations, the biological implications of our findings in thyroid carcinogenesis should also be discussed. Several oncogenic pathways have been implicated in the pathogenesis of papillary thyroid carcinoma, most notably the *BRAF* V600E mutation and *RET-PTC* translocations, two events that are mutually exclusive in general. DNA gene expression microarray studies have also shown that *BRAF* mutation-positive and mutation-negative papillary carcinomas are biologically distinctive in their mRNA expression profiles.⁴⁶ Morphologically, we and others have shown that classical papillary carcinoma cases are often *BRAF* mutation positive, whereas follicular variants are almost always *BRAF* mutation negative.^{47,48} In this regard, it is intriguing that miR-146b overexpression appears to be a common event in both classical and follicular variants of papillary carcinoma, likely irrelevant of the *BRAF* mutation (and probably the *RET-PTC* translocation) status. This finding implies that miR-146b overexpression is a late, rather than early, event in the pathogenesis of papillary thyroid carcinoma. Our finding that encapsulated follicular lesions with incomplete nuclear features of papillary carcinoma, possibly representing early lesions in transformation, lack miR-146b overexpression also supports this concept and would suggest that the downstream effects of miR-146b overexpression could be pivotal in establishing a complete carcinoma phenotype.

Although miR-146 was previously shown by microarray to be overexpressed in papillary thyroid

carcinoma, it was initially not possible to determine whether miR-146a or miR-146b was elevated based on hybridization-based microarray technique due to the minimal (2 of 22 base pairs) sequence difference between miR-146a and miR-146b. Confirming the findings of He *et al*,²¹ our results showed that miR-146b, but not miR-146a, was overexpressed in papillary carcinoma. Also, similar to previous studies,^{10,21,49} our results showed that the expression levels of miR-221 and miR-222 are closely coordinated. As both are clustered on X chromosome,⁵⁰ it is likely that they are encoded by a single polycistron, as was previously suggested.²² For diagnostic purposes, it would thus be unnecessary to assay both miR-221 and miR-222. In contrast, although miR-146b, -221, and -222 were all upregulated in papillary thyroid carcinoma, our data suggest that they are regulated differently in cancer. Consistent with this notion, miR-221 and miR-222 have been implicated in multiple tumor types,^{10,39,49,51–53} including glioblastoma and leukemia, and miR-146 has only been associated with papillary thyroid carcinoma and melanoma.^{8,21,22}

In summary, we showed that when compared to normal thyroid tissue, miR-146b, miR-221, and miR-222 were overexpressed in almost all cases of papillary thyroid carcinoma but not in follicular adenoma or hyperplastic thyroid nodules. Follicular lesions with only incomplete nuclear features of papillary thyroid carcinoma did not show miR-146b overexpression. These findings, coupled with the observation that miRNAs are well preserved in formalin-fixed paraffin-embedded tissues, suggest that differentially expressed miRNAs are promising targets in the molecular diagnosis of papillary thyroid carcinoma, and the potential value of miRNA analysis in the preoperative evaluation of thyroid nodules should also be further explored.

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