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RAPID COMMUNICATION

MicroRNA expression profile of thyroid nodules in fine-needle aspiration cytology: a confirmatory series

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Abstract

Introduction MiRNAs are small endogenous non-coding RNAs implicated with gene expression regulation. Changes in miRNA levels have been reported in thyroid cancer. Fine-needle aspiration cytology (FNAC) is the most reliable tool for differential diagnosis of thyroid nodules.

Methods We have analyzed 174 FNAC from 168 patients with thyroid nodules for expression levels of 11 miRNAs (miRNA197; -187; -181b-3p; -181b-5p; -224; -181a; 146b; -221; -222; -155 and miRNA183) known to be up-regulated in cancer tissues compared to benign lesions. Expression of miRNAs was analyzed in FNA samples calculating the fold change of miRNA expression relative to normal thyroid tissue after normalization to an endogenous control.

Results In FNAC, miRNA expression was confirmed to be higher in malignant or suspicious for malignancy nodules compared to benign, only for miRNA146b, -222 and -221 (fold change expression ≥ 5).

Conclusion In this study, we confirmed that a limited set of miRNAs can be used for the differential diagnosis of thyroid nodules.

Keywords Fine-needle aspiration cytology · miRNA221 · miRNA222 · miRNA146b · Thyroid cancer

Introduction

Thyroid nodules are very common in the general population, particularly in iodine deficient countries. The majority of thyroid nodules are benign hyperplastic nodules and thyroid cancer is found in less than 10%. Fine-needle aspiration cytology (FNAC) represents the gold standard to determine the nature of thyroid nodules. In expert hands, FNAC has an overall accuracy of 95%. The sensitivity is between 43 and 98% and the specificity is between 72 and 100%, with positive and negative predictive values of 89–98 and 94–99%, respectively [1].

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression. MicroRNAs may be involved in tumorigenesis acting both as tumor-suppressor genes and as oncogenes. The expression profile of miRNA in tissues seems to be useful in the discrimination between benign and malignant lesions. Different thyroid histotypes show a peculiar miRNA signature [2]. Our laboratory, have already contributed to establish, that in a large cohort of patients, serum miRNAs can be used to discriminate malignant from benign lesions. In particular, the expression levels of miRNAs-190 and -95 were combined in a mathematical formula resulting in a value, called pmiRNA, which estimated the risk of malignancy for each patient with a cut-off of 0.5. Values > 0.5 were correlated with an increased risk of malignancy. The pmiRNA showed a very high diagnostic power with an AUC% of 99.0% (95% CI 96.9–100%). However, the search of circulating miRNA may arise some problems such as the presence of cell-free circulating form together with exosome or protein-associated miRNAs which can alter their expression and may lead to different detection among laboratories. In this view, the analysis of miRNAs in cytological sample can be more promising.

Several recent studies [3–6] have used miRNA expression to differentiate benign from malignant thyroid nodules in FNAC. These studies reported a sensitivity and specificity ranging from 73 to 100 and 73.5 to 88%, respectively, considering a limited set of miRNAs.

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In light of these data, we aimed to validate miRNA expression utility in the differential diagnosis of thyroid nodules using the material recovered from FNAC.

Results

After a review of the literature [2–5], we selected a panel of 11 miRNAs (miRNA197; -187; -181b-3p; -181b-5p; -224; -181a; 146b; -221; -222; -155 and miRNA183) known to be up-regulated in thyroid cancer tissues compared to benign lesions. We collected 174 FNAC from 168 patients. At cytology, 7 (4%) were Bethesda categories III and IV and 16 (9.2%) were Bethesda I. Samples belonging to Bethesda I category were excluded from the study due to unsatisfactory or inadequate material. Samples belonging to Bethesda categories III and IV were analyzed separately. In the remaining 151, we calculated the fold change of miRNA expression in nodules with Bethesda categories II ($n=140$, 80%) and V–VI ($n=11$, 6.3%) relative to normal thyroid tissue after normalization to an endogenous control by real time q-PCR. Relative expression quantification was performed by the comparative cycle threshold (CT) method ($2^{-\Delta\Delta Ct}$). Expression of miRNAs was analyzed in FNA samples without knowing final histology. After surgery, 100% of the Bethesda categories V and VI studied were confirmed to be malignant (10 PTCs and 1 follicular variant of PTC). Once analyzed for their mutational status, all PTCs harbored the V600E BRAF mutation and the follicular variant showed the NRAS Q61R alteration. Cytologically benign nodules were not submitted to surgery.

In FNAC, miRNA expression was confirmed to be higher in malignant or suspicious for malignancy samples compared to benign only for miRNA146b, -222 and -221 ($p<0.001$ and $p<0.0001$, respectively) (Fig. 1). We restricted the analysis to these miRNAs and we observed that 10/11 (90.9%) malignant cases revealed more than fivefold expression of 2 (2/11, 18%) to 3 (8/11, 72.7%) miRNAs. One malignant sample had increased expression only for 1 miRNA of the panel and was considered a false negative result (Fig. 2a). Among the benign cases, 2 (1.4%) showed more than fivefold expression only for 2 miRNAs and were considered as false positive samples (Fig. 2a).

According to this cut-off, we obtained a sensitivity of 90.9%, a specificity of 98.5%, an accuracy of 98% with a positive predictive value of 84% and a negative predictive value of 99.3%.

We, then, performed the analysis on the 7 Bethesda III samples and IV. After the analysis of miRNA expression, 6 out of 7 (85.7%) indeterminate FNAC had a fold change less

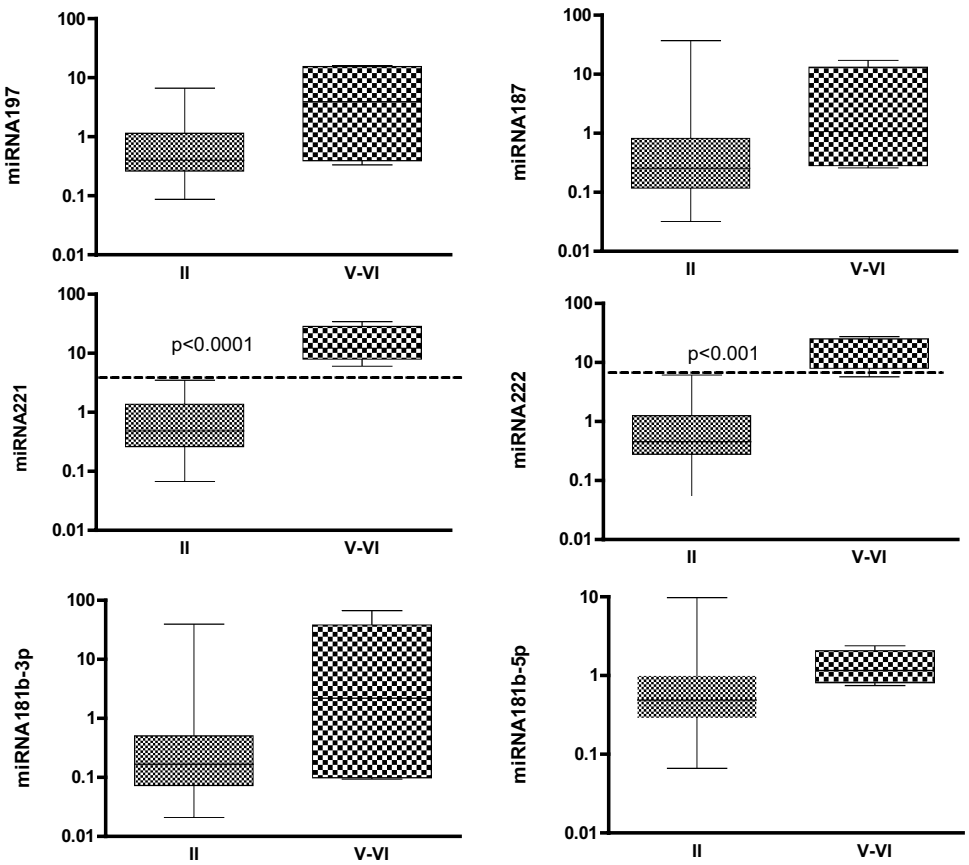
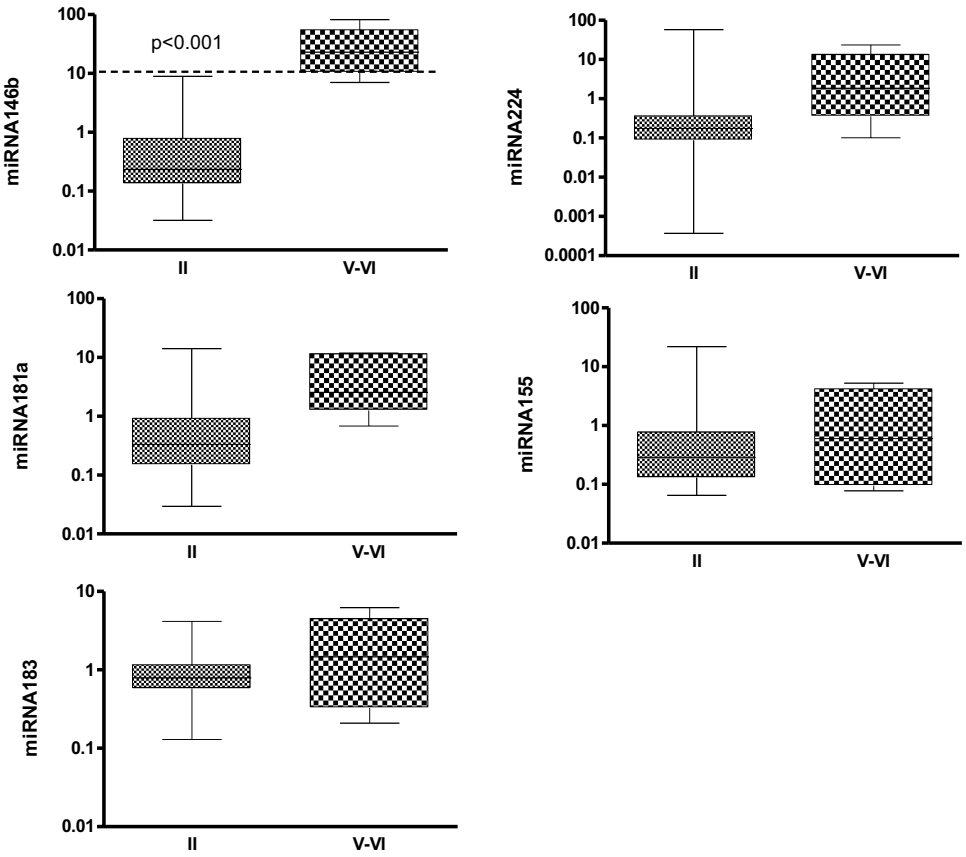


Fig. 1 Expression of 11 miRNAs in Bethesda categories II and V–VI relative to normal thyroid tissue after normalization to an endogenous control by real time q-PCR. Relative expression quantification was performed by the comparative cycle threshold (CT) method ($2^{-\Delta\Delta Ct}$) and data are reported as $\log_{10}$. Statistic was performed by Student's t test and $p<0.05$ was considered as significant. Dot line represents the overlap between cytologically benign and malignant cases

Fig. 1 (continued)



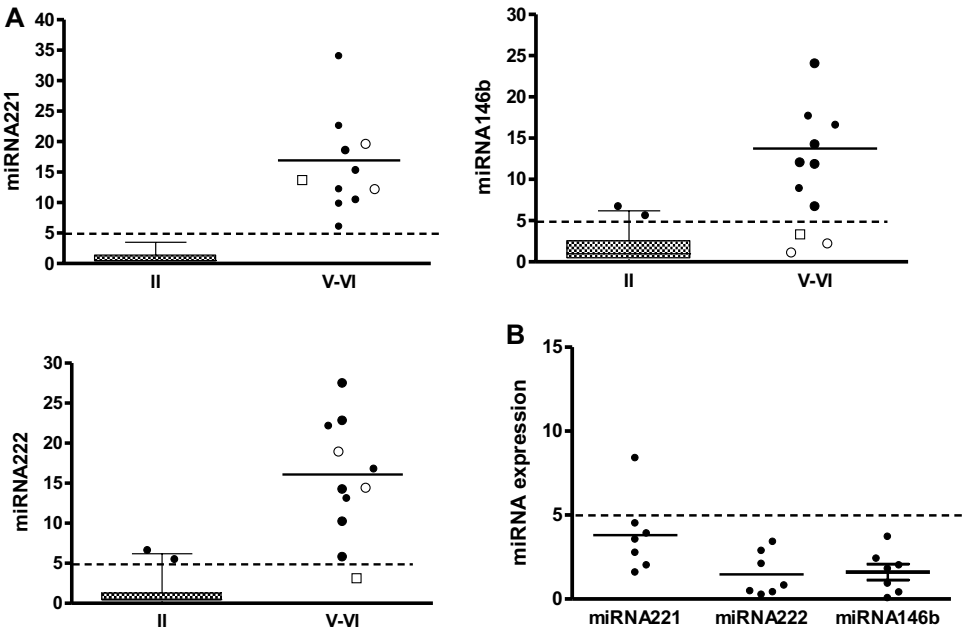


Fig. 2 **a** Graphical representation of false/true positive and false/true negative samples for miRNA221, -222 and -146b in Bethesda categories II and V–VI. Expression is reported relative to normal thyroid tissue after normalization to an endogenous control (linear scale). Dot line represents the cut-off of 5. Two benign samples were false positive with miRNA222 and 146b expression more than 5. One malignant sample was false negative with miRNA222 and -146b expression under the cut-off of 5 (white square). **b** Expression of miRNAs in Bethesda categories III and IV relative to normal thyroid tissue after normalization to an endogenous control (linear scale). Dot line represents the cut-off of 5

than 5 and 1/7 (1.4%) had an increased expression only for miRNA221 (Fig. 2b). According to our cut-off, these samples were classified as benign. In addition, we performed on indeterminate FNAC the search for genetic mutations such as BRAFV600E, hTERT promoter mutations, H–K-NRAS point mutations, RET/PTC and PAX8/PPARγ rearrangements (by PCR followed by DHPLC and direct sequencing). All samples were negative for these alterations and

follow-up was decided for 6 out of 7 (85.7%) patients. In these cases, nodules were stable at neck ultrasound and classified from low to intermediate risk according to ATA ultrasound risk classification. One patient was submitted to surgery for nodule dimension and histology documented a follicular variant of papillary thyroid cancer.

In summary, in agreement with other previous reports, in this paper, we confirmed that a limited set of miRNA may be useful for the preoperative diagnosis of thyroid nodules. In particular, differences in expression between benign and malignant nodules are more evident for miRNA221, -222 and 146b which seem to be useful also in the classification of indeterminate lesions. A multicentre study including a large number of nodules, especially of indeterminate lesions, is finally recommended.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with local ethical committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent has been required and signed by each participant to the study.

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