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Differential expression levels of plasma-derived miR-146b and miR-155 in papillary thyroid cancer

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SUMMARY

Background: Specific circulating microRNAs (miRNAs) in each organ may contribute to the diagnosis and prognosis in some cancers. miRNA from papillary thyroid cancer (PTC) may be released into the bloodstream. This study was performed to detect miRNAs in the plasma and estimate their diagnostic usefulness for discriminating between benign and malignant lesions.
Methods: Patients who underwent thyroidectomy for benign thyroid nodules or PTC were enrolled in this study. The patients were divided into three groups: benign, PTC without lymph node metastasis (LNM), and PTC with LNM. The levels of miR-146b, miR-221, miR-222, and miR-155miRNA expression in blood samples before surgery were evaluated.
Results: Of 89 patients enrolled in this study, 19 and 70 had benign lesions (21.3%) and PTC (78.7%), respectively. The mean levels of miR-146b and miR-155 expression were higher in the PTC group than the benign group. For discrimination between benign and PTC lesions, the area under the ROC curve (AUC) for miR-146b was 0.649 with 61.4% sensitivity and 57.9% specificity. The AUC for miR-155 was 0.695 with 74.3% sensitivity and 63.2% specificity ($P < 0.05$). The levels of miR-146b, miR-221, and miR-222 were slightly higher in the N1 group than the N0 group. The levels of miR-146b, miR-155, and miR-222 increased in proportion to tumor size.
Conclusions: miR-146b and miR-155 helped to discriminate between benign and malignant lesions. Circulating miRNA is likely a useful alternate serological marker for PTC. This preliminary study suggested that circulating miRNAs may be useful as follow-up tools as well as diagnostic tools.

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (19–25 nucleotides) that regulate gene expression at the transcriptional or post-transcriptional level [1]. Perfect or imperfect complementary miRNAs bind to the 3′ untranslated region (3′UTR) of an mRNA transcript and block its translation, thus leading to control of various cellular processes including cell differentiation, cell cycle progression, and apoptosis [2,3]. These multistep processes are associated with carcinogenesis via oncogenic or tumor suppressive functions. More than half of miRNA-related genes are located in the fragile regions that are mutated in malignant tumors, and miRNAs are associated with tumors [4]. Increased or decreased levels of miRNA expression have been observed in various types of cancers originating from the blood, lung, pancreas, breast and colon [5,6]. These results suggest that miRNAs may regulate the development, progression, and metastasis of malignant lesions.

Although ultrasonography (US)-guided fine needle aspiration (FNA) is widely used to diagnose thyroid nodules, the histological findings obtained using this method are frequently ambiguous, ranging from suspicious malignancy to atypia of undetermined significance [7]. Most cases of papillary thyroid cancer (PTC) have an exclusive genetic alteration that results in activation of various signaling pathways, including *BRAF, RAS, RET/PTC, and PAX8/PPAR* [8]. Therefore, these specific markers obtained from FNA may help clinicians to discriminate malignant from benign lesions [8]. These

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genetic alterations are also related to tumor phenotype and may predict tumor behavior and prognosis. However, these mutations do not always predict tumor prognosis and are not found in all cases of PTC. Therefore, individual underlying molecular changes must be elucidated to help differentiate benign from malignant lesions and to stratify tumor behavior and prognosis. In thyroid cancer, cell type and differentiation of the tumor affect the expression of different miRNAs. Increased expression levels of miR-221, miR-222, miR-21, miR-181a, and miR-146b were found in PTC compared with normal thyroid tissue [9]. Overexpression of miR-146, miR-221, and miR-222 were especially prominent in PTC [9,10], suggesting that dysregulation of these miRNAs plays a crucial role in this cancer type. In addition to miRNA-related tumorigenesis in thyroid cancer, altered expression of miR-146b is a prognostic factor, indicating *BRAF* mutation and an aggressive tumor pattern [11]. Most of the reports suggesting a clinical value of miRNAs were based on the findings from tissue specimen.

Because tumor-derived miRNAs are released into the bloodstream [12], specific types of circulating miRNAs in each organ may to contribute the diagnostic and prognostic roles in patients with certain types of cancer, such as lung, gastric, and colon cancer [13–16]. Some miRNAs, such as miR-21 and miR-26b have a diagnostic role in head and neck cancer [17]. We postulated that miRNAs expressed in PTC may also be released into the bloodstream. The present study was designed to detect miRNAs in the plasma and estimate their diagnostic usefulness for discriminating between benign and malignant lesions, or PTC with/without lymph node metastasis.

Materials and methods

Patients

Patients who underwent thyroidectomy for benign thyroid nodules or PTC between January 2009 and December 2010 were enrolled in this study. In addition to thyroidectomy, central lymph node dissection with or without lateral lymph node dissection was performed in PTC patients. This cross-sectional study was approved by the institutional review board (04-2012-016) and conducted in compliance with the Declaration of Helsinki. Blood samples were obtained before surgery with informed consent. The tissue specimens for this study were provided by the Pusan National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare, and Family Affairs. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

All pathological findings were confirmed after surgery, and PTC was diagnosed according to the diagnostic criteria to exclude variant types of PTC [18]. Patients with distant metastasis, any other malignancies and blood-related diseases were excluded. The enrolled patients were divided into three groups: benign, PTC without lymph node metastasis (LNM), and PTC with LNM, according to the TNM classification established by the American Joint Commission on Cancer (AJCC; 2010, 7th edition). Expression levels of the miRNAs detected in blood samples obtained before surgery were compared to determine clinical value.

Specimens and RNA extraction from plasma

Samples of peripheral venous blood (10 mL) were obtained for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis before surgery. Samples were collected in EDTA-containing tubes and stored at −80 °C. Total RNA was extracted from 500 μL plasma using the miRNA PARIS kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Virtually disturbed samples after centrifugation (lipemia and hemolysis) were excluded before RNA extraction to prevent a contamination of blood samples. All serum samples were checked for hemolysis, lipemia, and cellular contamination by complete blood cell count, potassium concentration, and visual check. If more than 20/mL of white blood cells, more than 2000/mL of platelets, or more than the upper reference value of potassium (5.5 mmol/L) was detected in serum samples, they were excluded. Double centrifugation was used to prevent cellular contamination. All serum samples were centrifuged for 20 min at 795*g* and 4 °C and the supernatants were moved to secondary tubes. An additional centrifugation for 20 min at 15,000*g* and 4 °C was done for each sample to remove platelets and their debris. Serum supernatants after the second centrifugation were collected and used for total RNA extraction.

Selection of miRNAs

Tissue-derived miR-146b, miR-221, miR-222, and miR-155 are elevated in of PTC, compared with normal thyroid tissue [19,20]. We selected these four miRNAs to determine whether their expression levels were elevated in plasma derived from PTC patients. Furthermore, we evaluated the expression levels of these markers according to tumor extent, with regard to extrathyroidal extension and nodal metastasis.

qRT-PCR analysis of miRNAs in plasma

The expression levels of individual miRNAs were measured using sequence specific primers (Applied Biosystems Inc., Foster City, CA, USA) and real-time RT-PCR-based detection. We used synthetic cel-miR-39 for quality control [21]. Synthetic cel-miR-39 (50 pmol) was added to 400 mL of each serum sample, and total RNA was extracted. We used two relative PCR quantitation methods to qualify miRNAs MiRNAs were quantified using TaqMan miRNA assays (Applied Biosystems) after reverse transcription (TaqMan MicroRNA RT Kit; Applied Biosystems) of 40 ng (plasma) RNA. To reduce the possible high intra-assay variance introduced by low abundance in miRNAs, a pre-amplification step using TaqMan PreAmp Master Mix (Applied Biosystems) was performed for all plasma RNA samples with cycling conditions: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The comparative cycle threshold (Ct) method was used for all target candidates (miR-146b, miR-155, miR-221, and miR-222) in this study, with serum samples. The synthetic cel-miR-39 of miRscript miRNA mimic (Qiagen, Valecia, CA) was added to all serum samples before total RNA extraction and was amplified in an identical way as the other candidate miRNAs. Delta Ct was calculated by subtracting the Ct values of cel-miR-39 from the Ct values of the targeted miRNAs. We used the standard curve method normalized with synthetic cel-miR-39 in the validation study to select the candidates (miR-146b, miR-155, miR-221, and miR-222). The standard curves were constructed with each synthetic miRNA and the miScript miRNA mimic. Standard references miRNAs were amplified for each reaction. The results were normalized to spiked cel-miR-39, which was added before total RNA extraction. Duplicate experiments were performed. Those Ct values above the determinable range (up to 45) were assigned a Ct of 45.

Statistical analysis

Statistical analyses were performed using SPSS software, version 18.0 (Chicago, IL, USA). The mean age of each group was compared using the Krusakal–Wallis test. The distributions of gender