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Research Article

DIAGNOSTIC VALUE OF CIRCULATING miRNA-16-5p AND miRNA-221-3p IN THYROID CANCER

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ABSTRACT

Objective: The aim of this study is to investigate the usability of microRNA 16-5P and 221-3P levels as biomarkers in the diagnosis of thyroid cancer.

Materials and Methods: Patients who underwent thyroid surgery due to suspicious thyroid nodules were included in the study. A total of 142 patients who agreed to participate in the study had 3-5 cc venous blood taken into EDTA tubes and biochemistry tubes before the operation. In the postoperative period, 68 patients with malignant pathology results and 74 patients with benign pathology results were grouped as the control group. *miRNA-16-5p* and *miRNA-221-3p* levels were measured in serum samples taken from the patients. The levels of miRNA levels in malignant and benign patient groups were analyzed.

Results: The miRNA-16-5p and miRNA-221-3p levels of malignant patients were statistically significantly lower than those of benign patients ($p<0.001$). The analysis showed that miRNA-221-3p and miRNA-16-5p values had diagnostic value in predicting thyroid malignancy. Using a cut-off value of 21.69 for miRNA-221-3p, ROC curve analysis detected 89.7% sensitivity and 71.4% specificity (AUC=0.779, $p<0.001$). Using a cut-off value of 15.34 for miRNA-16-5p, ROC curve analysis detected 32.3% sensitivity and 100% specificity (AUC=0.708, $p<0.001$).

Conclusion: It was observed that *miRNA-221-3p* and *miRNA-16-5p* values had diagnostic value in predicting thyroid malignancy and could be potential biomarkers.

Keywords: miRNA, Biomarkers, Thyroid cancer

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INTRODUCTION

Thyroid cancer is the most common endocrine malignancy. The most reliable method for preoperative detection of thyroid cancer is currently thyroid fine needle aspiration biopsy (FNAB). There is currently no reliable biomarker that can predict thyroid cancer before surgery (1). MicroRNAs (miRNAs), a class of small non-protein coding RNAs, contribute to the control of gene expression and affect vital processes such as development, cell differentiation, proliferation, and programmed cell death. More than half of miRNA genes are located in genomic regions commonly associated with cancer (especially fragile sites), highlighting their potential role in tumor development (2). Several studies have compared miRNA expression levels in cytological samples and pathological tissue samples from patients with and without papillary thyroid cancer and have revealed different miRNA expression profiles in thyroid cancer tissues (3–6). However, studies evaluating circulating miRNA levels in thyroid cancer patients are still insufficient. miRNA-16-5p is a microRNA that stands out with its tumor suppressor function in various malignancies. It is thought that miRNA-16-5p, which plays a role in basic biological processes such as cell cycle regulation, induction of apoptosis and suppression of proliferation, may also be effective in the pathogenesis of thyroid cancer with similar mechanisms (7). Some studies have shown that miRNA-16-5p levels are significantly reduced in papillary thyroid cancer patients, and it has been suggested that this reduction may be associated with tumor formation and progression (8). Therefore, evaluation of miRNA-16-5p levels may be valuable as a non-invasive biomarker in the early diagnosis of thyroid cancer. miRNA-221-3p is an oncogenic microRNA known to be expressed in many types of cancer. It has been shown that it may contribute to tumor growth by affecting gene pathways that promote cell proliferation and suppress apoptosis (9). Studies on thyroid cancer have shown that miRNA-221-3p levels increase, especially in papillary thyroid carcinoma tissues (10). However, some previous miRNA studies in thyroid cancer patients have yielded contradictory results. The prognostic value of miRNA levels in thyroid cancer and their usability as biomarkers are still controversial. In our study, we aimed to investigate the usability of circulating microRNA levels as biomarkers in the preoperative diagnosis of thyroid cancer.

MATERIALS AND METHODS

Patients who underwent thyroid surgery at Adnan Menderes University Faculty of Medicine Hospital between November 2019 and November 2020 were included in the study. 68 patients whose postoperative pathology results were malignant and 74 patients who had thyroid surgery for various indications and whose pathology results were benign were included in the study as the control group. A total of 142 patients were included in the study. Prior to the study, approval was obtained from the Adnan Menderes University Faculty of Medicine Clinical Research Ethics Committee (Date of Approval: 10.10.2019 Protocol No: 2019/120). After receiving ethics committee approval, the study was conducted on patients who were decided to undergo surgery and who had thyroid surgery at the Department of General Surgery. The patients and control group were given an explanation of the study's purpose and the procedures to be carried out. Written informed consent forms were prepared regarding the study and their written informed consents were obtained.

Patient selection

Prior to surgery, 3–5 cc of venous blood was collected from patients who consented to participate in the study, using EDTA and biochemistry tubes. Postoperatively, patients were classified as having either benign or malignant conditions based on their pathological findings.

Circulating miRNA expression analysis

Venous blood samples taken from patients were stored at -80 degrees. Samples were isolated using a miRNA-specific kit. miRNA isolation was performed using GeneAll, Hybrid-R miRNA (Cat no:325-150). miRBase database was used in primer design. After the samples were isolated, they were stored at -80°C again. It was performed using 142 miRNA and control primers (U6). Complementary DNA (cDNA) synthesis was performed with the obtained miRNA using WizScript™ cDNA Synthesis Kit (High Capacity) W2211. Complementary DNA (cDNA) synthesis was performed using stem-loop primers. Stem-loop primers specific to these miRNAs were designed. These primers are considered the most reliable method since they specifically bind to the miRNA sequence when synthesizing cDNA from miRNA (11). cDNA synthesis was performed using primers specific to each miRNA from the obtained miRNAs. Each sample was cDNAed with its own stem-loop primer. SYBR Green-based Real Time Polymerase Chain Reaction (PCR) was established from the obtained cDNAs. Samples were studied in 2 replicates.

Statistical analysis

Statistical analyses were performed using SPSS version 27 software. After the distribution structures of quantitative

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data were evaluated using the Kolmogorov-Smirnov test for normal distribution, independent samples t-test was used in independent groups for comparison of variables that provided the assumption of normal distribution between groups, and descriptive statistics were shown as mean±standard deviation. For quantitative variables that did not meet the assumption of normality, the Mann-Whitney U test or Kruskal-Wallis test was applied depending on the number of groups. Descriptive data were presented as median (25–75 percentiles). Chi-square test was used for analysis of qualitative data, and the results were given as percentages. Receiver Operating Characteristics (ROC) curve analysis was used to evaluate the diagnostic value of serum miRNA-16-5p and miRNA-221-3p levels in predicting thyroid cancer. For the identified significant cutoff values, corresponding sensitivity and specificity were calculated. It was considered statistically significant when the p value was <0.05.

RESULTS

The study included 142 patients scheduled for thyroid surgery at Adnan Menderes University Faculty of Medicine Hospital between November 2019 and November 2020. According to the postoperative pathology results, 68 of the patients were grouped as malignant (56 females, 12 males) and 74 as benign (61 females, 13 males). Both groups were similar in terms of gender (p>0.05). The median age of all patients was 50 (42-61). The median age of malignant patients was 48 (35.5-62), and the median age of benign patients was 52 (45.75-61), and no significant statistical difference was found between the two groups (p>0.05).

Table 1. Patient characteristics

	Malign (n=68)	Benign (n=74)
Age (median)	48 (35.5-62)	52 (45.75-61)
Sex	56 female 12 male	61 female 13 male
miRNA-16-5p (median)	17.61 (14.92-19.46)	19.32 (17.68-21.24)
miRNA-221-3p (median)	18.23 (16.30-19.84)	26.13 (20.55-28.67)

According to postoperative pathology results, 38 (55.9%) of the cases with thyroid cancer were diagnosed as micropapillary thyroid cancer, 27 (39.7%) as papillary thyroid cancer, 2 (2.9%) as thyroid medullary cancer, and 1 (1.5%) as follicular thyroid cancer.

miRNA-16-5p level was median 19.32 (17.68-21.24) in benign patients and median 17.61 (14.92-19.46) in malignant patients. miRNA-221-3p level was median 26.13 (20.55-28.67) in benign patients and median 18.23 (16.30-19.84) in malignant patients. miRNA-16-5p and miRNA-221-3p levels of malignant patients were statistically significantly lower than in benign patients (p<0.001).

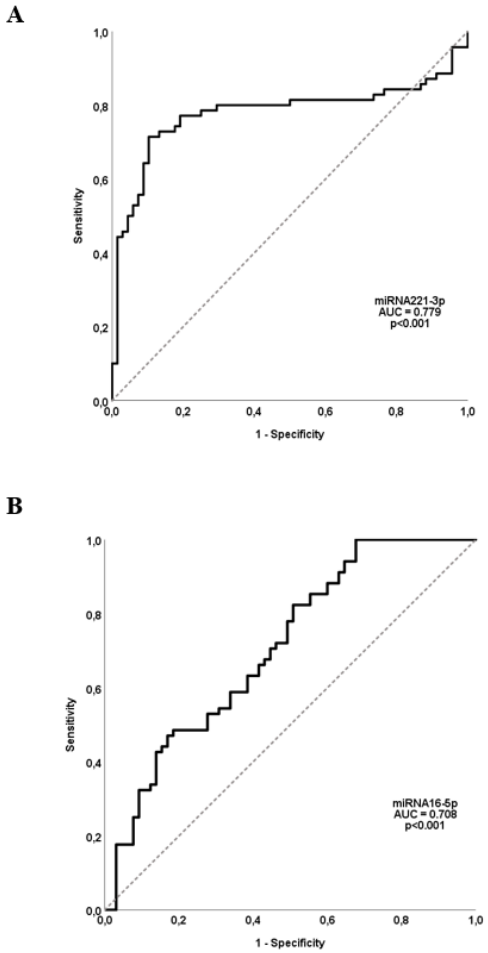

Figure 1. (A) miRNA-221-3p ROC curve. (B) miRNA-16-5p ROC curve.

When the echogenicity status was classified as hypoechoic, isoechoic and hyperechoic and miRNA-221-3p levels were examined, a significant difference was found between the 3 groups (p=0.011). When post-hoc analysis was performed, the miRNA-221-3p value in isoechoic patients was significantly higher than the values in hypoechoic patients (p=0.016). In patients with microcalcification, both miRNA levels were found to be lower than in patients without microcalcification (p=0.006 for miRNA-221-3p,

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Table 2. ROC Curve Analysis Results

	miRNA-221-3p	miRNA-16-5p
AUC	0.779	0.708
95% Confidence interval	0.700-0.845	0.623-0.784
Cut-off	21.69	15.34
Sensitivity	89.7%	32.3%
Specificity	71.4%	100%
p value	<0.001	<0.001

p=0.016 for miRNA-16-5p). There was no significant difference between the miRNA levels evaluated in our study and other parameters used in the distinction of malignant/benign in ultrasonography such as macrocalcification, halo, periphery calcification, nodule border pattern, nodule size and multinodularity (p>0.05). The analysis showed that miRNA-221-3p and miRNA-16-5p values had diagnostic value in predicting thyroid malignancy. Using a cut-off value of 21.69 for miRNA-221-3p, ROC curve analysis detected 71.4% specificity and 89.7% sensitivity (AUC=0.779, p<0.001). Using a cut-off value of 15.34 for miRNA-16-5p, ROC curve analysis detected 100% specificity and 32.3% sensitivity (AUC=0.708, p<0.001).

DISCUSSION

Thyroid cancer is becoming increasingly common worldwide. While it is usually indolent, it can be aggressive in certain patients. Diagnosis is typically achieved through ultrasound and fine-needle aspiration biopsy, but in some cases, distinguishing between benign and malignant nodules remains challenging.

In recent years, miRNAs have received growing attention due to their potential roles in cancer diagnosis, prognosis, and treatment response. Most studies have investigated miRNA expression in tumor tissues. However, this method is invasive and requires surgical intervention. In contrast, measuring circulating miRNA levels in serum offers several advantages: it is non-invasive, easy to perform, yields rapid results, provides preoperative diagnostic insight, and allows for serial monitoring. Despite these benefits, studies on circulating miRNAs have shown inconsistent results, which may be attributed to individual patient-related factors or external influences such as circadian rhythm (12).

Several studies have shown that miRNA-16-5p and miRNA-221-3p are involved in the molecular mechanisms of thyroid and other cancers. In our study, serum levels of miRNA-16-5p and miRNA-221-3p were significantly lower in malignant cases compared to benign ones. ROC curve analysis demonstrated their diagnostic utility in detecting thyroid malignancy, suggesting that these miRNAs may serve as valuable serum biomarkers.

miRNA-16-5p has been found to be downregulated in various cancers. For instance, its expression is reduced in osteosarcoma, and its overexpression inhibits cell migration, proliferation, and invasion (13). Ruan et al. reported that miRNA-16-5p expression was significantly lower in breast cancer tissues compared to non-cancerous tissues, with lower expression associated with higher tumor grade (14). Additionally, miRNA-16-5p was shown to inhibit cell proliferation and migration by targeting the actin-binding protein anillin in breast cancer cells (15). Similarly, Feng et al. observed downregulated miRNA-16-5p in PTC tissues compared to normal thyroid tissues (16). Our findings, which show significantly lower serum miRNA-16-5p levels in malignant thyroid nodules, align with previous reports of tissue-level downregulation. This supports the hypothesis that miRNA-16-5p plays a tumor-suppressive role. Importantly, our use of serum—rather than tissue—highlights the potential for a non-invasive, clinically applicable biomarker. However, further studies are needed to confirm whether serum levels accurately reflect intratumoral expression and whether they are sufficiently stable for routine clinical use.

The role of miRNA-221-3p in cancer remains complex, with varying findings reported. Fang et al. found lower levels of miRNA-221-3p in breast cancer tissues compared to normal tissues (17), and reduced expression was also associated with poorer survival in high-risk prostate cancer patients (18). Rogucki et al. supported its potential role in the diagnosis of PTC (19). Conversely, other studies have suggested an oncogenic function for miRNA-221. One study showed that miRNA-221 promotes proliferation and invasion in PTC cells by suppressing TIMP3 expression (20). Furthermore, both miRNA-221-3p and miRNA-222-3p were reported to be upregulated in thyroid cancer cell lines and were associated with treatment response (21).

However, findings on serum miRNA-221-3p levels have been inconsistent. Rosignolo et al. found no significant difference between serum miRNA-221-3p levels in PTC and benign nodule groups (22). Zhang et al. reported elevated serum levels in PTC patients compared to healthy controls, with associations to tumor location, extrathyroidal invasion, stage, and lymph node metastasis (23). Another study also found increased preoperative serum levels in PTC patients, though no correlation with clinical features was observed (24).

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In contrast, our study found that serum miRNA-221-3p levels were significantly lower in malignant cases. Using a cut-off value of 21.69, ROC curve analysis showed that miRNA-221-3p could predict thyroid malignancy with a sensitivity of 89.7% and specificity of 71.4% (AUC = 0.779, $p < 0.001$). These results support the potential use of serum miRNA-221-3p as a diagnostic marker.

One of the major factors contributing to inconsistencies in circulating miRNA levels across studies is the variability in RNA isolation techniques. The efficiency and purity of miRNA extraction from serum or plasma samples can vary significantly depending on the kits and protocols employed, directly impacting quantification results. Furthermore, pre-analytical variables such as sample collection, handling, and storage conditions are crucial in preserving miRNA stability. Another critical aspect is the choice of normalization strategy; while some studies utilize endogenous controls like U6 small nuclear RNA, others prefer exogenous spike-in controls such as cel-miR-39. These differing approaches can substantially affect data comparability and interpretation. This heterogeneity in RNA isolation and normalization methods may partly explain the conflicting reports regarding serum miRNA-221-3p expression in thyroid malignancy.

Interestingly, although our study found lower serum levels of miRNA-221-3p in malignant cases, several previous studies have reported increased expression of this miRNA, particularly in thyroid tumor tissues. For example, Zhang et al. demonstrated elevated serum levels in patients with papillary thyroid carcinoma. This discrepancy may arise from differences in sample types (serum versus tissue), ethnic variation, tumor heterogeneity, or methodological differences in RNA quantification. Additionally, the dynamic nature of circulating miRNAs—affected by factors such as degradation, exosomal release, and protein binding—may lead to contrasting serum levels compared to tissue expression. Therefore, further prospective studies are warranted to clarify the context-dependent expression patterns of miRNA-221-3p in thyroid malignancy.

This study has several limitations, including the small sample size, variability in blood collection timing, and the lack of postoperative miRNA follow-up measurements. Moreover, the limited cohort size precluded stratified analyses between thyroid cancer subtypes. To enhance the robustness and generalizability of these findings, future studies should incorporate larger, well-characterized patient populations, employ standardized blood sampling protocols, and implement longitudinal miRNA monitoring at clearly defined postoperative intervals.

CONCLUSION

Based on the findings of this study, miRNA-221-3p and miRNA-16-5p appear to have diagnostic value in predicting thyroid malignancy and may serve as potential biomarkers. Overall, the results of this study contribute to the growing body of evidence supporting the use of circulating miRNAs as novel biomarkers in thyroid cancer and may provide a foundation for future investigations in this area.

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Adnan Menderes University Faculty of Medicine Medical Oncology Clinic staff

Authorship contributions
EO, MBA and EG designed the study; EO, MBA, BD, İHE, NKÇ and MGÜ collected the data; İKÖ, MBA and EO carried out statistical analysis; EO, MBA performed the literature search; EG, MGÜ and EO supervised the study; MBA and EO prepared and revised the manuscript. All authors gave the final approval of the version to be published.

Data availibity statement
The data that support the findings of this study are available from the corresponding author, [E.O.], upon reasonable request.

Declaration of competing interest
The authors have no conflicts of interest to declare.

Ethics
Ethics committee approval was obtained from the Clinical Research Ethics Committee of Adnan Menderes University Faculty of Medicine Clinical Research Ethics Committee (Date of Approval: 10.10.2019 Protocol No: 2019/120).

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