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RESEARCH

The expression levels of MIR375 and MIR451a genes in subacute thyroiditis

Subakut tiroiditte MIR375 ve MIR451a genlerinin ekspresyon düzeyleri

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Abstract

Purpose: The most typical reason for thyroid pain is subacute thyroiditis (SAT). Incidence of SAT is higher in women than in men and the average annual frequency of SAT is 12.1 cases per 100,000 people. Endogenous, brief, single-stranded non-coding RNAs, called micro RNAs (miRNAs), are inhibitors of gene expression. In the posttranscriptional regulation of gene expression, miRNAs are essential. In this investigation, we explored the potential utility of two circulating miRNAs, MIR451A and MIR375, as markers for the detection and monitoring of SAT.

Materials and Methods: Fifty SAT patients and 41 healthy people were enrolled in this study. Using the quantitative real-time polymerase chain reaction (qRT-PCR) approach, miRNA expression levels were assessed. The $2^{-\Delta\Delta Ct}$ technique was used to calculate miRNA expression levels. The t-test was used to assess the statistical significance of miRNA expression.

Results: While MIR451A expression levels were discovered to vary between healthy controls and SAT patients, MIR375-3p expression levels were discovered to be similar in both groups. MIR375-3p and MIR451A expression levels were shown to be similarly in different SAT stages (hyperthyroid, euthyroid, and hypothyroid). The expression levels of MIR375-3p and MIR451A were analyzed for association with the clinical characteristics of SAT patients, but no correlation was observed.

Conclusion: In this study, it was found that the expression level of circulating MIR451A was lower in SAT patients, which shows that MIR451A might be effective in the SAT disease's onset.

Keywords:. expression level, subacute thyroiditis, MIR451A, MIR375.

Öz

Amaç: Tiroid ağrısının en tipik nedeni subakut tiroiditтir (SAT). SAT prevalansı kadınlarda erkeklere göre daha yüksektir ve yıllık ortalama sıklığı 100.000 kişide 12,1'dir. Mikro RNA'lar (miRNA'lar) adı verilen endojen, kısa, tek sarmallı, kodlamayan RNA'lar, gen ekspresyonunun inhibitörleridir. miRNA'lar gen ifadesinin transkripsiyon sonrası düzenlenmesinde önemlidir. Bu çalışmada, dolaşımdaki iki miRNA'nın (MIR451A ve MIR375) SAT'ın tespiti ve izlenmesinde belirteç olarak potansiyel faydasını araştırdık.

Gereç ve Yöntem: Bu çalışmaya 50 SAT hastası ve 41 sağlıklı birey dahil edildi. miRNA ekspresyon seviyeleri, kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) yaklaşımı kullanılarak değerlendirildi. miRNA ifade düzeylerinin hesaplamak için $2^{-\Delta\Delta Ct}$ tekniği kullanıldı. miRNA ifadesinin istatistiksel önemini değerlendirmek için t testi kullanıldı.

Bulgular: Sağlıklı kontroller ve SAT hastaları arasında MIR451A ekspresyon düzeyleri farklılık gösterirken, MIR375-3p ekspresyon düzeylerinin iki grupta da benzer olduğu belirlendi. MIR375-3p ve MIR451A ekspresyon düzeylerinin, farklı SAT evrelerinde (hipertiroid, ötiroid ve hipotiroid) benzer olduğu görüldü. MIR375-3p ve MIR451A'nın ekspresyon seviyeleri, SAT hastalarının klinik özellikleriyle ilişki açısından analiz edildi, ancak herhangi bir korelasyona rastlanmadı.

Sonuç: Bu çalışmada dolaşımdaki MIR451A'nın ekspresyon düzeyinin SAT hastalarında daha düşük olduğu bulundu, bu da MIR451A'nın SAT hastalığının başlangıcında etkili olabileceğini göstermektedir.

Anahtar kelimeler: ekspresyon düzeyi, subakut tiroidit, MIR451A, MIR375.

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INTRODUCTION

Thyroiditis is a condition that occurs when the thyroid gland becomes inflamed. One of the type of thyroiditis is subacute thyroiditis (SAT) and it is also called as giant cell thyroiditis, de Quervain's thyroiditis, painful thyroiditis, subacute nonsuppurative thyroiditis, or subacute granulomatous thyroiditis[1]. SAT is the most common reason of thyroid pain[2]. SAT usually develops after upper respiratory tract infections and is assumed to be due to a viral disease, although there is no conclusive evidence[1]. Neck stiffness or discomfort, palpable tenderness, and diseases like euthyroidism and hypothyroidism are common symptoms of SAT[3]. It is more common in women than in men, and the annual average incidence of SAT is, 12.1 cases per 100,000 people. It is mostly seen in young adulthood and middle age, and its incidence decreases with increasing age[4,5].

Endogenous small single-stranded non-coding RNAs, called micro RNAs (miRNAs), function in suppression of gene expression. They target mRNAs' 3' untranslated regions (UTR) for either cleavage or translational repression by selectively attaching to these areas[6]. Although their functions are not fully known, it is known that miRNAs have important roles in the regulation of cell differentiation, proliferation and survival[7]. Most human genes are expressed under the control of mature miRNAs, and it is thought that over 60 percent of human gene transcripts have at least one conserved miRNA binding site[8]. According to previous reports, miRNAs are crucial for immune system functioning and also for the appearance of autoimmune and autoimmunity diseases[9,10].

MIR375 is a gene that located on chromosome 2q35 and encodes a non-coding RNA (ncRNA). Functional analyzes of MIR375-depleted cells revealed greatly reduced neuroendocrine differentiation, anchorage-independent development of colonies resulting in much slower tumor growth in xenograft models[11,12]. MIR375 is known to control the precise maturation of cancerous cells in the lung, but it is unclear how it affects RNA editing and cancer development in carcinoids. Experimental findings showed that MIR375 behaves as an oncogene in H727 cells, despite the fact that it is widely believed to be a tumor suppressor in many malignancies[11,12]. Downregulation of MIR375 expression plays a role in the suppression of various types of cancer[13]. In the regulatory mechanism of MIR375, it can target many functional genes and regulate the transcription and translation of these genes. Therefore, it may be useful to use MIR375 as a new biomarker in the diagnosis of disease[14]. It has been observed that MIR375 is highly expressed in brain, colon, lung, stomach, small intestine and pancreas compared to other tissues[15]. This shows that MIR375 plays a regulatory role in many biological pathways. For example, MIR375 is effective in the regulation of the behavior immune cells (such as macrophages and helper T cells) in autoimmune diseases. MIR375 also contributes significantly to the growth of adipocytes, osteocytes, and neurons.

MIR451A is an ncRNA-coding gene located on chromosome 17q11.2. The miR-451 family contains two major members, identified as MIR451A and MIR451B, in the human genome. MIR451A is known to be highly involved in various cancers and it functions primarily as a tumor suppressor. MIR451A provides an important function in tumorigenesis and tumor development by being effective in changing the response to stress. According to studies, MIR451A stops tumor growth by inhibiting angiogenesis in human umbilical chord endothelial cells via the mechanism of vascular endothelial growth factor receptor 2 (VEGFR2) signaling[16].

Circulating miRNAs have been studied and applied as new biomarkers for a range of inflammatory and autoimmune disorders. MiR-155-5p has been demonstrated to be up-regulated in SAT patients, indicating that miR-155-5p might be a novel biomarker for SAT. As a result, the pathophysiology of SAT may be linked to the expression of miRNAs. In order to explore the possible utility of miRNAs as biomarkers for disease activity, we looked at the expression levels of immune-related miRNAs, MIR375 and MIR451A, in sera from patients with SAT.

MATERIALS AND METHODS

Sample

Fifty individuals who had been identified with SAT in the Endocrinology clinic of Medical Faculty of Tokat Gaziosmanpaşa University, were incorporated in this study. The diagnosis of SAT was made by an endocrinologist according to the 2016 American Thyroid Association guidelines, taking into account criteria such as ultrasonographic indications, physical examination, clinical picture and laboratory test

results. The presence of tender, painful and firm goiter, increase in free thyroxin (fT4), decrease in serum thyroid stimulating hormone (TSH), increase in C-reactive protein (CRP) or erithyrocyte sedimentation rate (ESR), decrease in vascularization appearance of painful thyroid zones on ultrasonography, and presence of hypoechoic areas with blurred borders were evaluated when making the diagnosis[17]. Fortyone applicants that applied to endocrinology clinic and did not have any chronic conditions especially thyroid disease, made up the control group. The consent form was signed by the participants and the patients' data were gathered using the created patient follow-up form. Tokat Gaziosmanpaşa University Clinical Research Ethics Committee granted the required approval for the study (18-KAEK-048) at its meeting on February 20, 2018.

Procedure

Whole blood taken into EDTA tubes from patients and healthy controls were first centrifuged at 3600 rpm for 10 minutes. Then, the plasma part of the blood was taken into Eppendorf tubes and kept at -80°C. RNA isolation was performed using RNA Isolation Kit (GeneAll Biotechnology, Seoul, Korea) from plasma samples by following the manufacturer. With the help of the cDNA synthesis mix included in the Single miR-qPCR Assay kit (A.B.T.™, Türkiye), cDNA conversion from the acquired RNAs was carried out. RT- PCR was performed from the obtained cDNAs with miR-qPCR Master Mix, which is also included in the Single miRNA qPCR Assay kit (A.B.T.™, Türkiye). The reverse transcriptase, RNase inhibitor, miRNA-specific stem-loop primer, antibody-mediated hot-start Taq DNA polymerase, forward and reverse primers, dNTPs, $MgCl_2$, SBYR green dye, ROX reference dye, enhancer, and stabilizer are all included in the miR-cDNA Synthesis Kit and miR-qPCR MasterMix to enable reverse transcription and RT-PCR reactions. RT-PCR was conducted using an Applied Biosystems 7500 Fast RT-PCR System (Applied Biosystems, Foster City, CA, USA). For this study, single miRNA qPCR Assay kits which were separately produced for MIR375-3p and MIR451A, and reference miRNA qPCR Assay kit (Rnu6) were used. All the laboratuary analyses were carried out in laboratories of Medical Biology Department of Faculty of Medicine in Tokat Gaziosmanpasa University.

The comparative threshold cycle (Ct) approach was used to determine the relative expression of each miRNAs. The Ct values of reference gene (Rnu-6) and the target genes were computated for this method. The fold change ($2^{-\Delta\Delta Ct}$) method was used to calculate the expression data of the studied miRNAs[18].

Statistical analysis

The statistical analyses were carried out using IBM SPSS Statistical Package Program Version 20.0 and Openepi 3.01 (www.openepi.com). The t-test was used to assess the statistical significance of miRNA expression. The characteristics of the patients were expressed as mean+standard deviation (SD) or numbers, depending on what was available. SAT patients' laboratory findings, such as serum fT4, free tri-iodothyronine (fT3), TSH, white blood cells (WBC), CRP, ESR and age, were compared with the expression levels of MIR375-3p and MIR451A using Pearson correlation analysis. All p values were two-tailed, and statistical significance was defined as a p value of 0.05 or less.

RESULTS

Table 1 lists the gender, age, thyroid hormone status, fT4, fT3, TSH, WBC, CRP and ESR values of SAT patients.

Table 1. Demographic and clinical characteristics of patients with SAT

Features	Patients (n=50)
Female gender, n (%)	40 (80.0)
Age, average±SD (years)	40.60±8.106
Thyroid hormone status, n (%)	
Hyperthyroidism	36 (72.0)
Euthyroid	8 (16.0)
Hypothyroidism	6 (12.0)
fT4, average±SD, ng/dL	1.97±1.081
fT3, average± SD, ng/L	4.58±2.870
TSH, average± SD, mIU/L	1.20±2.024
WBC, average± SD,10^3 /mm^3	11.00±9.264
CRP, average± SD, mg/L	50.04±49.282
ESR, average± SD, mm/h	59.32±25.480

The Pearson Correlation analysis was used for statistics. CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; fT4, free Thyrodine; fT3, free Triidothyron; SAT, subacute thyroiditis; SD, standard deviation; TSH, Thyroid Stimulating Hormone; WBC, White Blood Cell

Patients with SAT had a mean age of 40.60, and 80% of them were female. The control group's average age was 37.95, and 82.9% of them were female. Age and

gender differences between patients and controls were acceptable (p=0.273 and p=0.286, respectively). In this study, 12% of patients were in the Euthyroid stage, 16% of patients were in the Hypothyroid stage, and 72% of patients were in the Hyperthyroid stage.

The expression levels of two circulating miRNAs were detected in both the control and patient groups. The expression levels of MIR451A were found to be statistically lower in SAT patients than controls (p=0.049). When we compared the expression levels of MIR375, it was higher in controls than in patients, however this was not revealed a statistically significant association. The possible reason for this was the high standard deviation in the control group and the intersection of confidence intervals (Table 2).

Post-hoc power analysis was performed after the study. According to the results, 45.6% power was obtained for the MIR451A gene and 26.5% power was obtained for the MIR375-3 gene.

Table 2. Expression levels of MIR451A and MIR375-3p in SAT patients and controls

miRNA	Grup	N	Expression Level ($2^{-\Delta\Delta Ct}$)	p Value
MIR451A	SAT Patients	50	5.77±14.2	0.049
	Controls	41	16.41±34.53	
MIR375- 3p	SAT Patients	50	11.19±48.9	0.145
	Controls	41	105.99±453.4	

The T-Test was used for statistical analysis. SAT, subacute thyroiditis.

No statistically significant associations were detected for either of the expression levels of two circulating miRNAs (MIR451A and MIR375-3p) when we compared each patient stages (hyperthyroid, euthyroid, and hypothyroid) with the control group and with each other seperately (Tables 3 and 4; Figure 1) (p > 0.05).

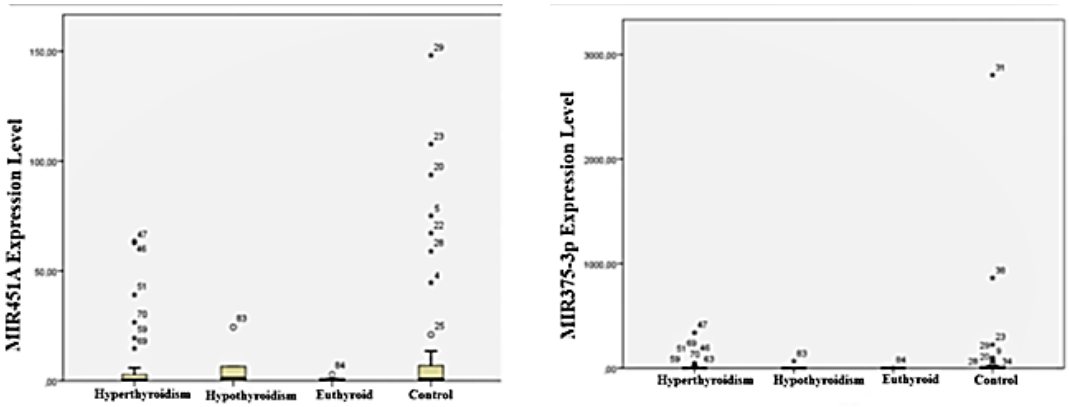

Figure 1. Comparison of MIR451A and MIR375-3p expression levels in plasma from SAT patients at various stages (hyperthyroid, euthyroid, and hypothyroid) and controls.

Table 3. Expression levels and p values of MIR451A in various disease stages of SAT patients and controls

Group	N	Expression Levels ($2^{-\Delta\Delta Ct}$)	p values					
			Hyperthyroid vs. Hypothyroid	Hyperthyroid vs. Euthyroid	Hyperthyroid vs. Control	Euthyroid vs. Hypothyroid	Hypothyroid vs. Control	Euthyroid vs. Control
Hyperthyroid	36	6.95±16.170	0.999	0.923	0.376	0.985	0.769	0.390
Hypothyroid	6	5.57±9.570						
Euthyroid	8	0.64±0.953						
Control	41	16.41±34.534						

The ANOVA test was used for statistical analysis. SAT, subacute thyroiditis.

Table 4. Expression levels and p values of MIR375-3p in various disease stages of SAT patients and controls

Group	N	Expression Levels ($2^{-\Delta\Delta Ct}$)	p values					
			Hyperthyroid vs. Hypothyroid	Hyperthyroid vs. Euthyroid	Hyperthyroid vs. Control	Euthyroid vs. Hypothyroid	Hypothyroid vs. Control	Euthyroid vs. Control
Hyperthyroid	36	13.58±56.670	1.000	1.000	0.561	1.000	0.898	0.813
Hypothyroid	6	11.62±26.860						
Euthyroid	8	0.10±0.168						
Control	41	105.99±453.392						

The ANOVA test was used for statistical analysis. SAT, subacute thyroiditis.

Table 5 and 6 respectively displays the findings of correlation studies between the expression levels of MIR451A and MIR375-3p and the clinical features (age, serum TSH, fT4, fT3, WBC, ESR, and CRP) of SAT patients categorized according to thyroid hormone status (hyperthyroid, hypothyroid, and euthyroid). Following this analysis, no correlation was observed ($p>0.05$) (Table 5, Table 6).

Table 5. Correlation analysis between clinical characteristics of SAT patients classified by thyroid hormone status and expression levels of MIR451A gene

Features	Total SAT patients (n=50)		Hyperthyroid (n=36)		Hypothyroid (n=6)		Euthyroid (n=8)	
	r	p	r	p	r	p	r	p
Age	0.039	0.785	-0.025	0.884	0.294	0.572	0.235	0.575
TSH	-0.018	0.906	0.071	0.689	0.217	0.726	-0.38	0.93
fT4	-0.017	0.912	-0.081	0.65	-0.235	0.704	-0.164	0.698
fT3	-0.083	0.706	-0.135	0.592	-0.267	0.733	-	-
WBC	0.039	0.803	0.278	0.137	0.076	0.903	-0.198	0.323
ESR	0.111	0.471	0.064	0.733	-0.101	0.872	0.396	0.331
CRP	-0.216	0.176	-0.28	0.114	-0.357	0.556	0.977	0.137

The Pearson Correlation analysis was used for statistics.CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; fT4, free Thyrodine; fT3, free Triidothyron; r, correlation coefficient; SAT, subacute thyroiditis; TSH, Thyroid Stimulating Hormone; WBC, White Blood Cell

Table 6. Correlation analysis between clinical characteristics of SAT patients classified by thyroid hormone status and expression levels of MIR375-3p gene

Features	Total SAT patients (n=50)		Hyperthyroid (n=36)		Hypothyroid (n=6)		Euthyroid (n=8)	
	r	p	R	p	r	p	r	p
Age	0.197	0.175	0.195	0.255	0.531	0.279	0.581	0.171
TSH	-0.041	0.785	-0.073	0.682	0.202	0.745	-0.233	0.616
fT4	0.1	0.945	-0.035	0.846	-0.22	0.722	0.043	0.927
fT3	-0.124	0.573	-0.151	0.549	-0.266	0.734	-	-
WBC	0.297	0.056	0.323	0.82	0.92	0.883	-0.161	0.731
ESR	-0.001	0.995	0.126	0.5	-0.082	0.896	0.547	0.204
CRP	-0.179	0.263	-0.211	0.24	-0.388	0.518	0.123	0.921

The Pearson Correlation analysis was used for statistics.CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; fT4, free Thyrodine; fT3, free Triidothyron; r, correlation coefficient; SAT, subacute thyroiditis; TSH, Thyroid Stimulating Hormone; WBC, White Blood Cell

DISCUSSION

Thyroiditis is a condition that occurs when the thyroid gland becomes inflamed. The most typical reason for thyroid pain is SAT[2]. It usually develops after upper respiratory tract infections and is assumed to be due to a viral disease, although there is no conclusive evidence[1].

miRNAs, which were first discovered in C. elegans in the early 1900s, are ncRNAs that have a high degree of conservation. They are about 22 nucleotides lenght[19]. miRNAs typically act as post-transcriptional negative regulators of gene expression[20].

In mouse researches, mice with chronic hypothyroidism were shown to have higher expression levels of several miRNAs, whereas mice with hyperthyroidism was shown to have lower expression levels of some miRNAs. Significant differences ($p<0.05$) were found in mRNA and miRNA levels for 92 transcripts with known functions between euthyroid and hypothyroid mice. Among them, the expression level of the miR-206 gene was found to be quite high in thyroidit group compared to the control group. This shows us that the expression levels of miRNA genes in mice can vary according to the stage of thyroiditis[21]. In another study, it was observed that certain miRNA expression levels were lower in a significant portion of tumor cells in the thyroid compared to normal cells. In addition, some miRNAs (miR-222, miR-146b, and miR-221) were found to be quite active in tumor cells in the thyroid tissue. In that study, it was seen that the expression levels of different miRNAs in tumor cells expressed in thyroid tissue are also different. The expression level of miRNA-181 is quite high in thyroid tumors, but it has been observed that miRNA-181 is expressed lower in tumors carrying the B-raf proto-oncogene (BRAF) gene mutation[22]. He et al. discovered the presence of active miRNAs (miR-146, miR-221/222, miR-155, miR-34 and miR-181) that are highly expressed in tumor tissue and played a role in the pathogenesis of Papillary thyroid carcinoma (PTC). Studies on PTC cells have shown that miR-221 was expressed at a high level in PTC cells, but at a low level in normal thyroid tissue. It has also been observed that overexpression of miR-221 may lead to the formation of PTC[23].

As a result of our study, which was carried out with a total of 91 participants, 50 SAT patients and 41 healthy controls, it was observed that the expression level of the MIR451A gene showed a significant difference between SAT patients and healthy control group ($p = 0.049$). The expression level of MIR451A in SAT patients was found to be significantly lower than in healthy controls. In our previous study, no correlation was observed between SAT and the expression levels of MIR16-1-3p and MIR22-3p genes[24]. Nonetheless, in our previous study, we found a relationship between the plasma MIR22-3p and MIR16-1-3p expression levels and the clinical features of SAT patients which was not observed in the current study. In our literature review, we encountered an extra study that examined the connection between SAT and miRNAs[25]. According to results of this study, expression levels of miR-155-5p was elevated in SAT patients. In contrast to this result, we observed lower expression of MIR451A in SAT patients in our study. This suggests that MIR451A and miR155-5p might both contribute to the pathophysiology of SAT.

In another study investigating the relationship between serum miRNA levels and autoimmune thyroid diseases (AITD), Graves' Disease and Hashimito's disease, it was shown that those with Graves' disease had higher expression levels of MIR16, MIR22, MIR375, and MIR451A genes[26]. On the other hand, patients with Hashimito disease had higher levels of MIR22, MIR375, and MIR451A expression compared to healthy people. Serum MIR375 levels have been found to be higher in people with AITD than in control participant[26]. In the current study, however, we could not find a relationship between expression levels of MIR375 and SAT, but we found an inverse relationship between expression levels of MIR451A and SAT. It was also previously observed that people with Graves' disease have decreased levels of the expression of genes such miR-154, miR-376b, and miR-431.

Thyroid hormone receptor beta (THRB) can be inhibited by increased expression of various miRNAs, including miR-221, miR-146a, miR-21, and miR-181a[27]. In another study, it was shown that miRNA-208a has an effect on thyroid hormone, and miRNA-208b and miRNA-499 have an effect on myofiber type determination and energy metabolism in skeletal muscle[28]. Angiotensin type 1 receptor (AT1R), one of the target genes of miRNA-350, was known to be involved in thyroid hormone-induced cardiomyocyte hypertrophy (thickening of the heart muscle wall). This situation causes the expression level of some miRNA (miRNA-208a and miRNA-

133) genes to change[28]. It was known that the expression levels of miRNA-17 and miRNA-92 genes were high in individuals with anaplastic thyroid cancer (ATC)[29]. Frisk et al. showed that inactivation of PTEN, a tumor suppressor gene, may be effective in the formation of malignant tumors and thyroid cancer. It has been determined that high expression levels of miRNA-19a and miRNA-19b are both closely related to inactivation of PTEN and associated with malignant tumor and thyroid cancer[29].

SAT has a male/female incidence rate of 1/4.3 and has an annual incidence rate of 4.9/10 million. SAT is most commonly seen in women between the ages of 30-50 and constitutes 0.5-6.2% of all thyroid diseases[30]. In our study, the male to female ratio was one-fourth, with a mean age of 40.60 years. It was observed that the age and gender distribution of our SAT patient group was similar to the available data.

According to the literature review, no study examining the relationship between the expression levels of MIR451A and MIR375-3p genes and SAT was found. This makes our work unique. Numerous miRNAs can express themselves at varying levels in different thyroid stages, and different miRNAs (miR-146b, miR-222, miR-221, and miR-206) express themselves at different levels in different thyroid cancer cases and thyroid tumors. It was thought that many clinical features such as thyroid hormone, fT3, fT4, WBC, ESR may be effective in the low expression level of MIR451A. The expression levels of MIR451A and MIR375-3p were also analyzed for a correlation with the clinical traits of SAT patients, but no correlation was observed.

The small number of all participants (SAT patients and controls) and the few number (only two) of circulating miRNAs that were analyzed are the limitations of the current investigation. Being the first study to examine the connection between SAT and the expression levels of the MIR451A and MIR375-3p genes, is the study's strongest point.

In conclusion, our study demonstrated that circulating MIR451A expression levels were decreased in SAT patients, indicating that MIR451A may contribute to the pathophysiology of SAT. We think that this investigation about the MIR451A and MIR375 gene expression levels in SAT, will serve as a foundation for future research into this condition as well as numerous other illnesses (such as cancer and diabetes). Also results regarding expression levels of MIR451A could be supported by cell line studies.

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