The Egyptian Journal of Hospital Medicine (January 2023) Vol. 90 (2), Page 2500-2508

# What about The Role of *miRNA125a-5p* in Iraqi Patients with Autoimmune Hashimoto's Thyroiditis?

**Satae Y. Mohammed*, Haidar J. Muhammed**
Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq
**Corresponding author:** Satae Y. Mohammed**, E-mail:** sthg@uomustansiriyah.edu.iq

## ABSTRACT

**Background:** Hashimoto's Thyroiditis a common disease that affects the thyroid gland. Recent studies on Hashimoto's Thyroiditis indicated the ability of miRNA to silence messenger RNA and regulate post-transcriptional processes. **Objective:** The aim of the current study was to find the relationship between microRNA 125a-5p and the production of antibodies thyroid peroxidase (Anti-TPO). **Patients and methods:** The present study enrolled 125 patients, whom 25 were males and 100 females; the ratio of males to females was 1:4. Blood samples were collected from the participants from the Specialized Center for Endocrinology and Diabetes, Rusafa, Baghdad (Iraqi Ministry of Health). Hashimoto's patients were diagnosed using clinical methods, ultrasound, and serological testing. Also, anti-TPO were detected using the ELASI technique. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was used to detect microRNA125a-5p levels in 40 samples from patients with Hashimoto's disease and compare them with 10 healthy individuals.

**Results:** There were high levels of Anti-TPO in patients with Hashimoto's disease compared with the healthy individuals, with a statistically significant difference ($P \leq 0.01$). The study also showed a decrease in the levels of miRNA 125a-5p in patients with Hashimoto's disease compared with the healthy individuals, with a statistically significant difference ($P \leq 0.01$). **Conclusion:** The study showed a decreased *miRNA-125a-5p* expression that was found to be inversely associated with the low level for gene expect. This could be related to many factors as patients might be taking medication, population differentiation, races, miRNA, and drug interaction.

**Keywords:** Thyroid hormones, autoimmune Hashimoto's disease, thyroid peroxidase antibody (Anti-TPO), miR125a-5p, Comparative study, Mustansiriyah University.

## INTRODUCTION

Hashimoto's thyroiditis is caused by a complex multistep process including genetic, environmental, and immunological variables accompanied by a decrease of thyroid hormones T3 and T4. In a nutshell, the loss of immunological tolerance to normal thyroid cells drives the generation of antibodies against thyroid tissue, resulting in thyroid gland death. When genetically predisposed individuals are exposed to the aforementioned environmental variables, the illness process begins with inflammatory changes [(1)]. As in autoimmune thyroid illness, the epitope recognition pattern of antibodies is changed, initiating immunological and inflammatory processes [(2)]. As well as important thyroid peroxidase, an enzyme that catalyzes the oxidation of iodine also plays a significant role as an autoantigen in the disease pathogenesis [(3)].

The production of auto-reactive cells directed against the thyroid gland in which immunological tolerance loss has been linked to genetically determined immune abnormalities or a lack of regulatory T-cells, which serve as suppressors [(4)].

Antibodies thyroid peroxidase (Anti-TPO) autoantibody detection is the most sensitive of the three anti-thyroid autoantibodies for diagnosing chronic thyroiditis [(5)]. miRNA is a small single-stranded non-coding RNA molecule that functions in silencing and post-transcriptional gene expression regulation. miRNAs have recently been identified as powerful regulators of many genes and pathways involved in the pathogenesis of inflammatory and autoimmune diseases [(6)]. Non-coding RNAs are well-known to play crucial roles in the pathogenesis of HT. Which binds to the 3'-untranslated regions (3'-UTRs) of messenger RNAs (mRNAs) in a sequence-dependent manner and ultimately either represses translation or causes degradation of target mRNAs [(7,8)]. Molecular RNA has been linked to various human diseases and is pursued as a therapeutic target and as a clinical diagnosis [(9)].

Their mean length is about 20 bp and used in clinical research and medicine in a slew of recent studies [(10,11)]. The apparent high stability of cell-free miRNA afforded by small size and packing in supramolecular complexes is one of the explanations for this trend [(12)].

According to recent research, evidence is mounting that microparticles are implicated in the etiology of autoimmune disorders and are instrumental in directing immune responses. Recent research has discovered that miRNAs play a role in T and B cell proliferation, maturation, and differentiation, and thus may influence the outcome of the immune response [(13)].
The aim of the current study was to find the relationship between microRNA 125a-5p and the production of Anti-TPO.

## PATIENTS AND METHODS

The study enrolled 125 patients, whom 25 were males and 100 females; The ratio of males to females was 1:4.



Received: 02/09/2022
Accepted: 05/11/2022

The clinical symptoms of patients in this study were diagnosed by clinical features, ultrasound, and laboratory tests that included tests for thyroxine (T3) (3.28–6.47 pmol/L), and thyroxine (T4) (7.64–16.03 pmol /L), thyroid stimulation hormone (TSH) (0.56-5.91 uIU/ml) by Cobbs C311, while immunoassays included Auto-TgAb and Auto-TPOAb by Enzyme-linked immunoassay (ELISA) MyBioSource/ USA, using the indirect method.

**Micro-RNA analysis:**

A total of 40 samples were selected from total samples that indicated an increase in the level of TSH and a decrease in T4 and T3, and elevated levels of anti-thyroperoxidase and anti-thyroglobulin antibodies, and healthy control group included 10 individuals, and the levels of mentioned tests were normal.

**RNA Extraction and qRT-PCR**

Total RNA was isolated using the Lysis buffer from Thermo Scientific Company, USA. The micro-RNA was extracted according to the manufacturer's instructions using Chloroform to get the upper aqueous phase containing the RNA and for RNA precipitation, isopropanol was added. The RNA pellet was washed by adding 70% ethanol to isolate RNA from other components then the microRNA pellet was rehydrated in 20-50µl by nuclease-free water in a heat block or water bath set at 55–60°C, for RNA solubility. The determination of RNA yield by fluorescence method was used.

**Synthesis of the cDNA from mRNA**

**First-strand cDNA synthesis and reaction component**

Using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit, total RNA was reverse-transcribed to complementary DNA (cDNA).

According to the manufacturer's instructions, the operation was performed in a reaction volume of 20 µl. (4µl) of total RNA had to be reversely transcribed as shown in **Table 1**.

**Table (1): Strand cDNA synthesis reaction component.**

| Component | Volume reaction |
|---|---|
| **mRNA** | 4 µl |
| **Anchored Oligo(dT)18 Primer (0.5 µg / µl)** | 1 µl |
| **Random Primer (0.1 µg / µl)** | 1 µl |
| **GSP** | 1 µl/ 10 pmol |
| **2xES Reaction Mix** | 10 µl |
| ***EasyScript*® RT/RI Enzyme Mix** | 1µl |
| **gDNA Remover** | 1µl |
| **RNase-free Water** | 1µl |
| **Total volume** | 20µl |

**Expression levels of *miRNA 125a-5p***

The expression levels of the *miRNA 125a-5p* gene were performed by the qRT-PCR SYBR Green test to estimate the expression of the target gene. Endogenous control gene *U6* mRNA levels were amplified and utilized to normalize the *miRNA 125a-5p* gene levels. **Table 2** shows the *U6* and *miRNA 125a-5p* genes primer sequences.

**Table (2) shows the *U6* and *miRNA 125a-5p* genes primer sequences.**

| Primer | Sequence (5′→3′ direction) | primer size bp | Ta °C |
|---|---|---|---|
| **miR125a-5p- (RT)** | GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCTGCAG | **46** | |
| ***miR125a-5p*** | | | |
| Forward | CGATCCCTGAGACCCTTTAA | 20 | 58 |
| Reverse | TATGGTTTTGACGACTGTGTGTGAT | 25 | |
| ***miRU6*** | | | |
| **F.P.** | AGAGAAGATTAGCATGGCCCCT | 22 | 58 |
| **miRNA-universe** | | | |
| **R.P.** | GCGAGCACAGAATTAATACGAC | 24 | 58 |

**Quantitative Real-Time PCR (qRT–PCR) runs:**

The Quantitative Real-Time PCR (qRT–PCR) was carried out using the QIAGEN Rotor gene Q Real-time PCR System (Germany). The expression levels and fold changes of *miRNA 125a-5p and miRU6* genes were assessed by measuring the threshold cycle (Ct) using the *TransStart*® Top Green qPCR Super Mix kit components. Every reaction was done twice. The needed volume of each component was calculated according to **Table 3**.

**Table (3): Quantitative real-time PCR components were used in *miRU6* and *miRNA125a-5p* gene expression experiments.**

| Components | 20 µl rxn |
|---|---|
| *2xTransStart*® Top Green qPCR Super Mix | 10 |
| Nuclease free water | 6 |
| Forward Primer (10 µM) | 1 |
| Reverse Primer (10 µM) | 1 |
| cDNA | 2 |

According to the thermal profile, the cycling protocol was programmed for the following optimized cycles, as given in **Table 4.**

**Table (4): The thermal profile of the *miRU6* gene and *miRN125a-5p* gene**

| Step | Temperature (ºC) | Time (sec.) | Cycles |
|---|---|---|---|
| Enzyme activation | 94 | 30 | 1 |
| Denaturation | 94 | 5 | 40 |
| Annealing | 58 | 15 | |
| Extension | 72 | 20 | |
| Dissociation | 55 ºC-95 ºC | | 1 |

**Calculation of *miRNA125a-5p* genes expression**

The double delta Ct (threshold cycle) analysis was used to assess the expression of *miRNA125a-5p* genes, in which *miRNAU6* were the housekeeping reference genes.

The calculations were as the following: By using the real-time cycler software, the threshold cycle (CT) was calculated for each sample.

All samples were run in duplicate and mean values were calculated. The Ct values for the housekeeping genes (*miRNAU6*) and the target genes (*miRNA125a-5p*) being tested in patients and controls were recorded.

**Ethical Considerations: All procedures for obtaining samples from patients were approved by the Department of the Specialized Center for Endocrinology and Diabetes of the Baghdad Health Department, Rusafa, after obtaining consents from the participants. All procedures in this study adhered to standard biosecurity and occupational safety procedures.**

***Statistical Analysis***

The collected data were introduced and statistically analyzed by utilizing the Statistical Package for Social Sciences (SPSS) version 20 for windows. Qualitative data were defined as numbers and percentages. Quantitative data were tested for normality by Kolmogorov-Smirnov test. Normal distribution of variables was described as mean and standard deviation (SD), and independent sample t-test/ANOVA test was used for comparison between groups. P value ≤0.05 was considered to be statistically significant.

**RESULTS**

**Table 5** summarizes and compares the age of 4 studied groups and the control group.

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**Table (5): Age of tested groups compared with the control group.**

| Age/Year | Minimum | Maximum | Mean | Std. Error | Std. Deviation |
|---|---|---|---|---|---|
| **Control** | 22 | 36 | 28.8 | 0.99 | 4.41 |
| **Group 1** | 17 | 78 | 50.42 | 1.87 | 14.94 |
| **Group 2** | 20 | 70 | 49.23 | 2.19 | 12.95 |
| **Group 3** | 24 | 66 | 49 | 3.45 | 12.92 |
| **Group 4** | 28 | 55 | 47.75 | 2.15 | 7.45 |
| Significant difference P-value ≤0.05 /NS=non-significant.<br>P-value between tested groups and Control: 0.001.<br>P-value between tested groups: 0.73 (NS). | | | | | |

The results of the current study revealed that individuals with Hashimoto's disease had significantly higher total serum anti-TPO levels than healthy controls as shown in **Table 6.**

**Table (6): Comparison of Anti-TPO levels in patients and healthy.**

| Anti-TPO N.V= less than 0.295 | Minimum | Maximum | Mean | Std. Error | Std. Deviation |
|---|---|---|---|---|---|
| **Control** | 0.11 | 0.21 | 0.16 | 0.01 | 0.02 |
| **Group 1** | 0.13 | 0.76 | 0.42 | 0.02 | 0.14 |
| **Group 2** | 0.11 | 0.73 | 0.38 | 0.03 | 0.17 |
| **Group 3** | 0.21 | 0.75 | 0.43 | 0.03 | 0.13 |
| **Group 4** | 0.21 | 0.73 | 0.41 | 0.04 | 0.15 |
| Significant difference P-value ≤0.05 /NS= non-significant.<br>P-value between tested groups and Control: 0.01 (significant).<br>P-value between tested groups: 0.5 (Not significant). | | | | | |

While the miRNA 125a-5p gene expression and housekeeping gene were measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and a relative quantification method.

The Ct value and the folding method (2-ct) were used to quantify gene expression after normalizing it to the level of the housekeeping genes (RNAU6) **(14).**

**The expression level of *miRNAU6***

**Quantification of *miRNAU6* expression by Real-time PCR**

The Ct value of *miRNAU6,* the housekeeping gene was used in this study, presented in **Table 7**. The Ct values for *miRNAU6* ranged from 20.27 to 20.20 in patients and healthy, respectively. These results indicate that there is no significant difference in the *miRNAU6* Ct value between these groups (P= 0.915). In addition, the variance in the overall change in *miRNAU6* expression in the different study groups was analyzed using the 2-C value and the 2-C ratio of the different study groups to the control group.

**Table (7): Expression level of *miRNAU6.***

| Group | Means Ct of *miRU6* | $2^{-Ct}$ | experimental group/ Control group | Fold of gene expression |
|---|---|---|---|---|
| **Patient** | 20.27 | 7.909E-07 | 0.952638 | 0.952 ± 0.27 |
| **Control healthy** | 20.20 | 8.302E-07 | 1 | 1 ± 0.00 |
| **P-value** | -- | -- | -- | 0.915 NS |
| NS: Non-Significant. | | | | |

**Comparison of *miRU6* Fold expression between study groups:** The calculated ratios for the expression of the *miRNAU6* gene fold in the patient group were 0.952, while the calculated ratios for the gene fold in the healthy control group were 1.00. Statistical indications are that there is a very slight difference between the examination group and the healthy control group. The *miRNAU6* gene acts as an appropriate control gene. Plots of *miRNAU6* gene amplification and dissociation curves are shown in **Figures 1 and 2**.

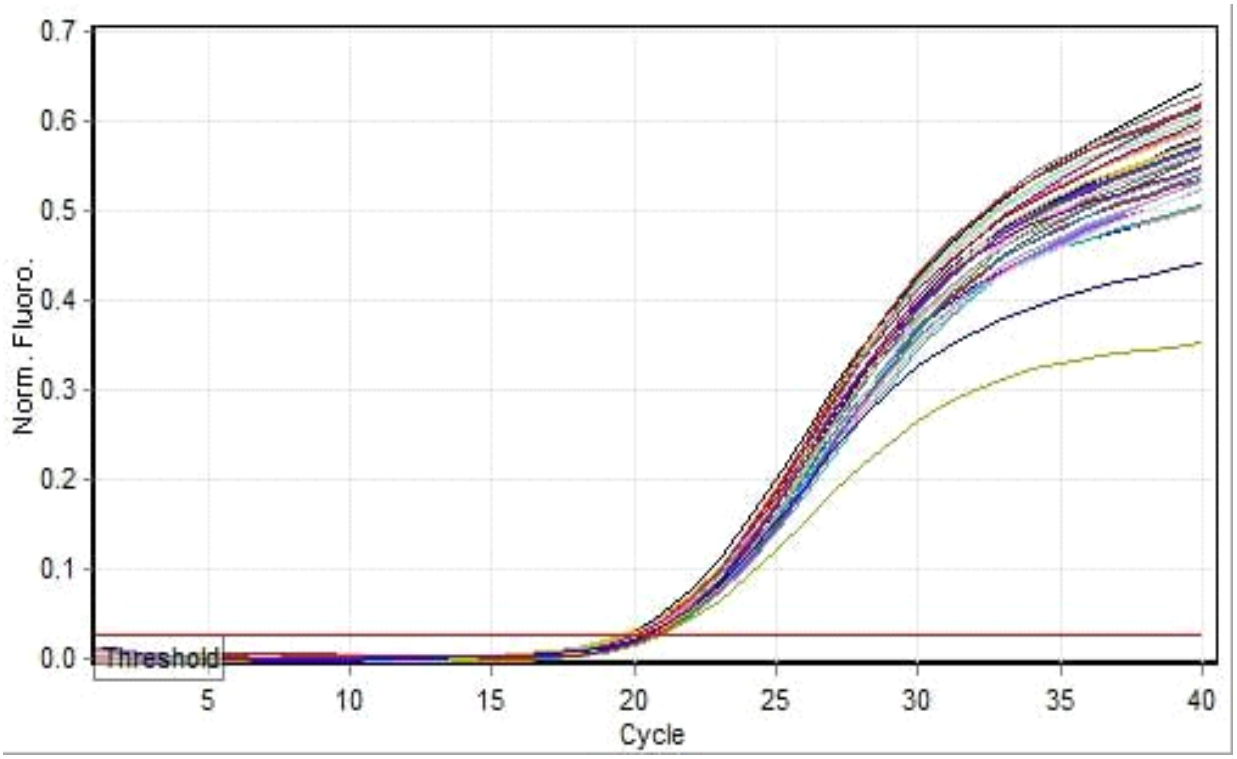


**Figure (1): Standard Curve plots of *miRU6* gene amplification and dissociation curves.**

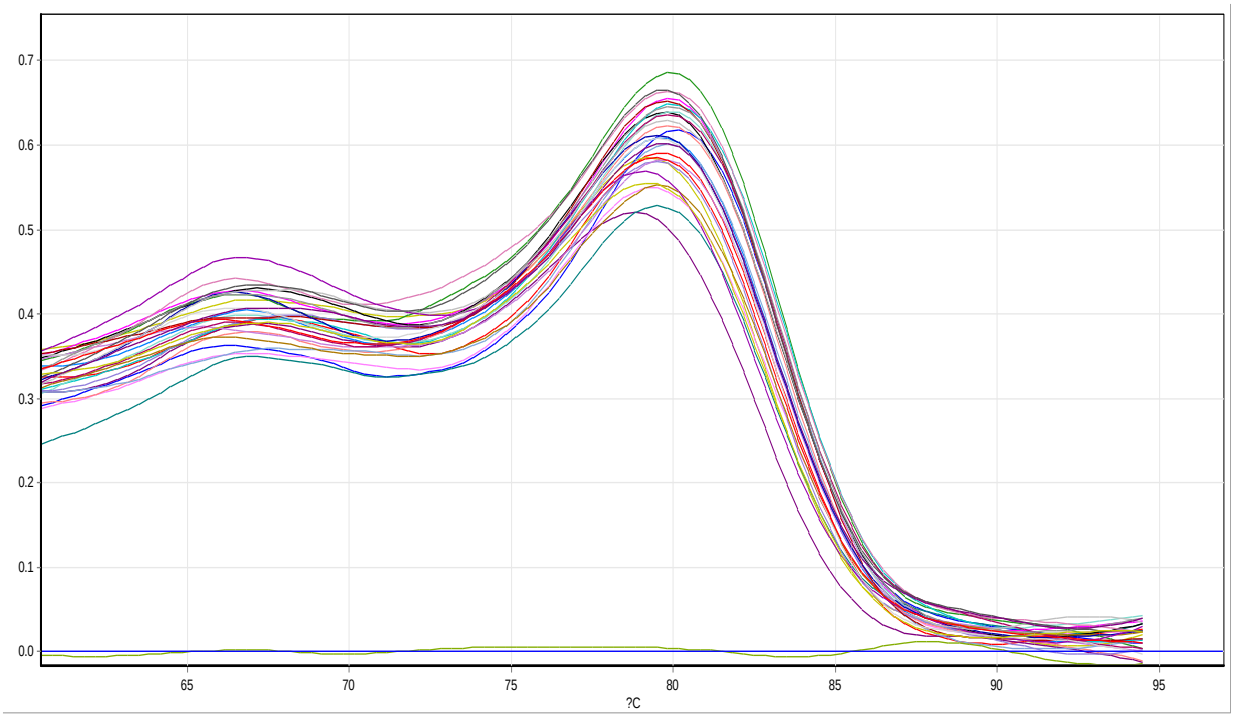


**Figure (2): The melting of melting *miRU6.***

The statistical results showed that there were statistically significant differences between the test group patients and the healthy control group **(P≤0.05**) which the cuff tilted to control as indicated through the table below, explained that the folding levels of *miRNA125a-5p* in the patients were low (**0.048 ± 0.01)**, while indicated. It was higher in the healthy control group (**1 ± 0.00)** as shown in **Table 8**.

The samples of patients were examined to the Ct values which were 29.49 and the ΔCt values were 9.223, while the Ct values of the healthy control group and the Ct values were 25.05 and the delta ΔCt was 4.856. The results indicated low readings in the group of patients examined, indicating low levels of expression of *miRNA-125a-5p*, while the results of the CT scan of the healthy control group were high, indicating high levels of expression of miRNA125a-5p, as shown in **Table 8** and **Figures 3** and 4.

**Table (8): Comparison of the *miRNA125a-5p* gene in Ct, ΔCt values between investigation groups.**

| Groups | Means Ct of *miRNA125a* | Means Ct of miRU6 | ΔCt | $2^{-\Delta Ct}$ | experimental/ Control group | Fold gene expression |
|---|---|---|---|---|---|---|
| **Patient** | 29.49 | 20.270 | 9.223 | 0.002 | 0.048 | 0.048 ± 0.01 |
| **Control healthy** | 25.05 | 20.200 | 4.856 | 0.035 | 1.000 | 1 ± 0.00 |
| **P-value** | -- | -- | -- | -- | 00 | 0.0392 * |
| * (P≤0.05). | | | | | | |

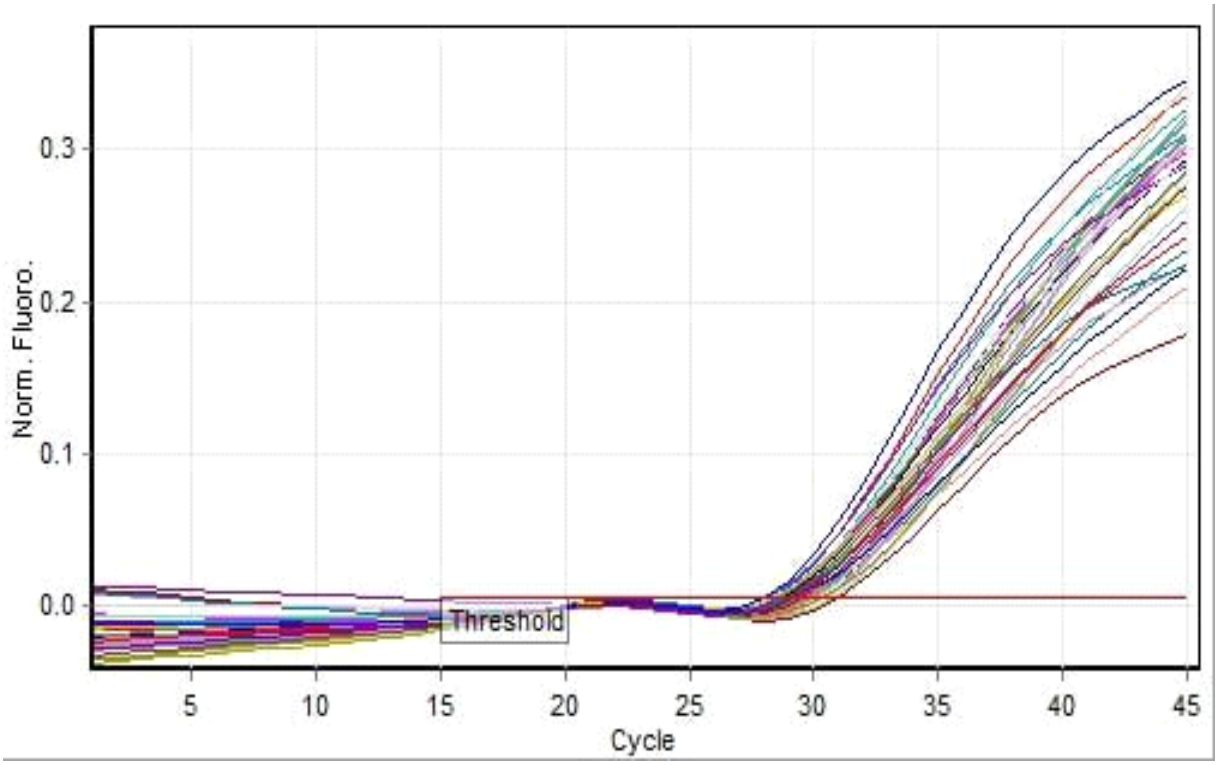


**Figure (5): Quantification of *miRNA* 125a-5p expression by Real-time PCR.**

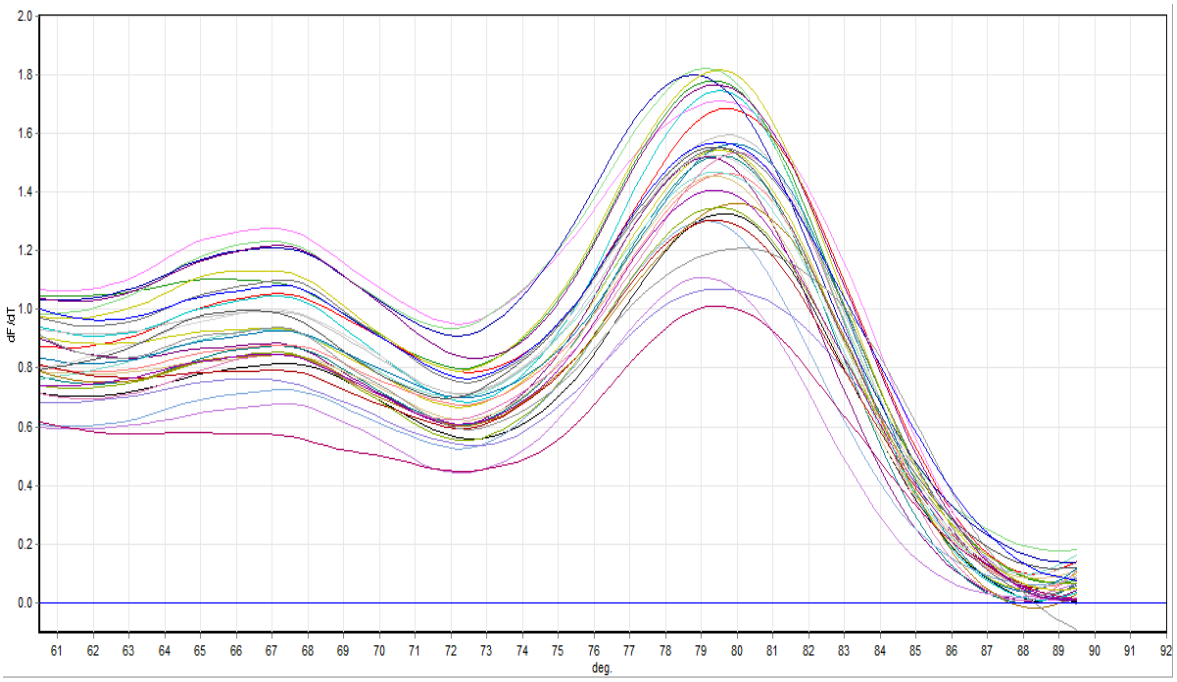


**Figure (6): Standard melting curve *miRNA125a*-5p.**

## DISCUSSION

### Total serum anti-TPO levels:

The results of the current study are in agreement with an Oxford University study by **De Leo and Pearce (2018)** showing that elevated anti-TPO values are commonly associated with Hashimoto's disease [15]**.** These findings are also consistent with another study conducted by **Ralli and colleagues** showing that higher rates of anti-TPOs are associated (95%) with Hashimoto's thyroiditis [16]**,** and another study found that people with Hashimoto's hypothyroidism had higher levels of Anti-TPO antibodies in serum compared to healthy subjects with normal levels of anti-TPO in adults (0-0.34 IU/ml) [17]**.**

Also, this was indicated by another study issued by the National Academy of Medical Sciences, Bear Hospital, Kathmandu (Nepal). This was a cross-sectional retrospective study in which antibody rates greater than 0.34 were considered positive [18]**.**

Another study indicated the early appearance of anti-TPO antibodies before the onset of thyroid disorders and hormonal disorders of TSH and T4 [19]**.** It is possible that this study differs from a study conducted by the American Thyroid Association in that about 5% of patients diagnosed with Hashimoto's thyroiditis based on a clinical diagnosis or by ultrasound do not have positively measurable thyroid antibodies. Another study conducted by **Czarnocka and colleagues** indicated there are 2 types of immune mechanisms for the destruction of thyroid cells by anti-peroxidases (anti-TOP); First by antibody-dependent cytotoxicity, including killer (NK) cells, and second by C3-dependent cytotoxicity (CDC) [20]**.** Previous studies by Smith and colleagues showed that helper T cells produced during disease activate B lymphocytes (plasma cells) and then produce antithyroid (anti-TPO) and anti-thyroglobulin (anti-Tg) antibodies that target thyroid cells [21].

**The expression level of *miRN125a-5p* and miRNAU6 genes:** Micro-RNAs (miRNAs) are small noncoding RNA molecules that organized gene expression after stages of transcription and depression translational of gene-related or segregation by targeting the three untranslated regions (UTRs) in that specific messenger RNAs (mRNAs) transcribed [22]**.** In the current study, we attempted to demonstrate the effect of *microRNA- 125a-5p* in the pathogenesis of Hashimoto's hypothyroidism. Examinations conducted on the test patients group and the healthy control group normalized by *miRNAU6 gene*. The study indicated that dysregulated (down) expression of miRNAs 125a-5p in Hashimoto's hypothyroidism as mentioned above contrasted with have focused on miRNA studied in plasma and thyroid tissue [23,24]**.** As well as by **Liu and colleagues (2022)**, who had declared upregulation of *miRNA -125a-5p* in the blood of Hashimoto's thyroiditis that indicated the first study explained the extraction of miRNA from blood HT disease [25]**.** However, little is known about the expression of microRNA and unclear profiles and the role of miRNAs in the peripheral blood related to extraction from thyroid-disordered patients.

A study published by **Otmani and Lewalle** indicated that miRNA is down-regulated in cancer cells and acts as a suppressor that's mean tumor suppressor or oncogenic depending on related mutated genes [26]**.** According to the research presented by **Liu and colleagues (2020)**, high-expression microRNA affects Th1 regulation by affecting the *MAF* of a related gene, a transcription factor that suppresses Th1 activity in Hashimoto's thyroiditis [27]**.** Thus, an affection in myocardial *miR-125a-5p* overexpression inhibited *MTFP1* expression and could stimulate the wild-type *MTFP1* promoter luciferase activity [27,28]**.**

The current study indicated an inverse association with low levels of a related gene and according to the mentioned study microRNA is a major factor in the pathogenesis of Hashimoto's hypothyroidism [29]**,** and the results of our study in reversely with **Liu and colleagues** [27] as shown in our results. These results can be explained by the use of tested mi RNA in the blood of collected samples which may give a lowering in levels of micro-RNA in the tested patient's group related to HT compared with normal control. While many studies suggested that serum and plasma should be used in trying the measurement of *miRNA -125a -5p* [21,30,31]**.**

Or corresponding with other studies that talked about the drug effects that patients used through the period of injury this cleared by principle pharmacogenomics explained the role of miRNAs in significant effect in downregulation of genes related so are important for drug function as well as/or through interactions consisting of a miRNA and a drug associated with the gene in which affects the biological activities of the pathways interactions. In which a connection of miRNA expression and drug function by combining the miRNA targeting and protein-associated drug interactions [32]**.**

The gene may in which predicted to be expressed and targeted by more than one miRNA that affected the deregulation of one of them. Also, studies explained multiple miRNAs have deregulated in the same tissue as well as in one disease, and drug resistance may therefore affect the combined actions of many miRNAs which can act on different genes. Such conditioning may be diagnosed through 'overlapping associations. As for a cancer cell line resistance to cisplatin has been associated with the down-regulation of four different genes by seven different miRNAs [33,25]**.** On the other hand miRNA deregulation supposedly initiates a signaling setup of the cascade which can be used in secondary pathways and at

the same time have the effects that lead to the deregulation of several genes, as suggested in a study reported by **Giovannetti and colleagues [34].**

In other probability in population differences in the taking of account, complicated features may be contributed to the combined effects of the social economic, environmental-genetic factors, and epigenetic aspects. Population differences can be observed for many complicated features including drug sensitivity **[35].** A phenomenon that has been termed pharmaco-ethnicity, although genome-wide surveys have observed molecular genetics in genes and chromosome variation contributing to gene expression **[35,36].**

## CONCLUSION

The study showed a decreased *miRNA-125a-5p* expression that was found to be inversely associated with the low level for gene expect. This could be related to many factors as patients might be taking medication, population differentiation, races, miRNA, and drug interaction.

**Conflict of interest:** The investigators declare no conflict of interest.
**Sources of funding:** The current study didn't receive any specialized grant from funding agencies.

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