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# ASSOCIATION OF CIRCULATING MICRO-RNAs WITH CLINICAL FEATURES IN IRAQI PATIENTS WITH GRAVES DISEASE

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**Abstract**

MicroRNAs (miRNAs) play an important role in the development and functions of the immune system and involved in the pathogenesis of autoimmune diseases mainly by regulating gene expression. Circulating miRNAs can be used as a diagnostic and therapeutic target for a variety of autoimmune diseases. This study aims to assess the existence of any possible association between the expression of circulating miR-146a-5p, miR-142-3p, and let-7b with clinical features of Graves disease (GD).

Forty patients with GD and forty healthy controls were involved in this study. Patients were divided into groups based on the presence or absence of clinical features (goiter and/or orbitopathy). The expression of circulating miR-146a-5p, miR-142-3p, and let-7b was determined by two steps Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique. Results obtained show that there is a significant elevation ($p<0.01$) in the expression of miR-146a-5p, miR-142-3p and let-7b in serum of patients compared with the control group, while there is non-significant overexpression ($p>0.05$) in patients with goiter and patients with orbitopathy compared to patients without goiter and orbitopathy. It was found that miR-146a-5p and miR-142-3p were positively correlated with the level of thyroid-stimulating hormone receptor antibody (TSHR-Ab), while let-7b is negatively correlated.

In conclusion; the up-regulation of miR-146a-5p, miR-142-3p and let-7b in patients and the positive correlation with TSHR-Abs indicating that circulating miRNAs could be used as biomarkers and targets for treatment, however, there is no association with its clinical features.

## INTRODUCTION

Autoimmune thyroid diseases (AITDs) are the most prevalent autoimmune disorders in humans, although AITDs have common features such as marked female preponderance, shared susceptibility alleles, and common auto-antigens, GD and autoimmune hypothyroidism have contrasting clinical characteristics [1]. Graves disease is characterized by diffuse hyper-functional goiter usually of recent onset that related to an immunological thyroid stimulation factor [2]. And it is caused by TSHR-Ab which binds to the TSHR on the surface of thyrocytes and abnormally activates the thyroid gland resulting in uncontrolled overproduction of thyroid hormones causing hyperthyroidism [3]. Extra thyroid manifestations also occur; mostly graves orbitopathy (GO) with or without dermopathy which is the most common cause of adult exophthalmos that occurs in about 50% of patients [4]. Graves's orbitopathy is correlated with an elevated level of TSHR-Ab and severe hyperthyroidism [5].

The exact mechanism of GD pathogenesis is not clear, it was believed that it is due to the combined effect of genes, environmental factors and the immune system [6]. Epigenetic modifications caused by environmental factors may drive genetically susceptible individuals to develop diseases, major epigenetic mechanisms include DNA

methylation, histone modifications, and noncoding RNAs play a pivotal role in the development and functions of the immune system mainly by regulating gene expression [7]. MicroRNAs are conserved endogenous, short non-coding RNAs that regulate gene expression through post-transcriptional processing by binding primarily to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) resulting in degradation and/or translational inhibition of mRNA [8]. Understanding the role of miRNAs can provide important and novel information about disease pathogenesis and the patient's clinical condition, since circulating miRNAs may regulate various biological processes, including immune functions, cell apoptosis, differentiation, and bioactivities related to intercellular communications [9].

Alteration in the expression of miRNAs has been reported in various immunological disorders, and circulating miRNAs have been an interesting area of research due to their stability and reproducibility [10] and due to their potential role as novel biomarkers and therapeutic targets [11]. MiR-146 plays a central role in inflammatory responses by modulating the transcriptional activity of nuclear factor kappa B (NF-κB) [12]. It can up-regulate IL-6 production by direct targeting of *Notch2* gene which involves in the proliferation, differentiation, and functions of various immune cells such as Th-cells, therefore miR-146a can promote the progression of inflammatory diseases [13]. Alterations in the expression of miR-146a affect the function of T-regulatory cells (Tregs) by targeting signal transducer and activator of transcription-1 (STAT1) which also leads to the differentiation of Th1 cells and IFN-γ production, therefore it could modulate the Th1-dependant inflammatory response [14]. MiR-142-3p also impairs the function of Tregs by reducing the production of cyclic adenosine monophosphate (cAMP) that required for suppressor function of Tregs by targeting adenylate cyclase 9 (AC9) mRNA [15].

In AITDs miR-142 directly target claudin-1(*CLND1*) gene, and its overexpression in thyrocytes can impair the human thyroid epithelial barrier function by down-regulating *CLDN1* expression resulting in increased permeability of thyrocytes monolayer which allows the exposure of thyroid auto-antigens to the immune system [16]. While let-7 modulates the inflammation by stimulating IL-6 signaling which activates STAT3-dependent NFκB transcription, and it induces the differentiation of pathogenic Th1 and Th17 cells by targeting IL-10 mRNA [17]. Although there are many studies have investigated the association of miRNAs in the thyroid tissue of patients with AITDs, few studies focused on their clinical and diagnostic utility [11]. This study aims to assess the existence of any possible association between the expression of circulating miR-146a-5p, miR-142-3p, and let-7b with clinical features of GD.

## MATERIALS AND METHODS

This case-control study conducted during the period from August to December 2019. Blood samples were collected from forty patients with GD (9 male and 31 female) attending Baghdad center for radiotherapy and nuclear medicine with age range (20-50 years). The diagnosis of patients was confirmed by specialists based on clinical features (goiter and/or orbitopathy), hormonal study; free T3 (FT3), free T4 (FT4), thyroid-stimulating hormone (TSH) and positive TSHR-Ab. Patients were divided into groups based on the presence or absence of clinical features (32 patients with goiter vs 8 patients without goiter) and (30 patients with orbitopathy vs 10 patients without orbitopathy). The control group included forty healthy subjects with normal levels of FT3 (2.17 – 3.34 pg/ml) [18], FT4 (0.82 – 1.63 ng/dl) [19], and TSH (0.38–4.31 μIU/ml) [20]. Patients and controls are free from any other autoimmune diseases were asked for their permission to give blood samples and signed informed consent for their agreement to participate in this study. The study was ethically approved by the College of Medicine/University of Baghdad and the Ministry of Health in Iraq.

### Measurement of TSHR-Abs

Thyroid-stimulating hormone receptor antibody was measured by indirect Enzyme-Linked Immunosorbent Assay (ELISA) using TSHR-Ab ELISA Kit (Demeditec/Germany). The absorbance of (< 1.2) which is equivalent to an antibody concentration of (>1.5 U/L) is considered positive **[21].**

### MicroRNAs Extraction

Total RNA including miRNAs were extracted from serum samples using Trizol reagent supplied by Ambion Life Technologies, USA. The homogenization of each sample was done by the addition of 1ml of Trizole reagent to 0.4 ml serum, and then stored at -20 ºC until used. The extraction procedure was described by Hummon et al. **[22]**. RNA concentration and purity were determined by measuring the absorbance at 260nm and 280nm using Nanodrop spectrophotometer.

### Two Steps Reverse Transcription Polymerase Chain Reaction

The expression level of circulating miRNAs was determined by RT-PCR technique. Reverse transcription involve conversion of the extracted miRNAs into cDNA using TaqMan MicroRNA reverse transcription kit and reverse transcription primers specific for each miRNA supplied by Applied Biosystems/Thermo fisher scientific, USA, and it was carried under thermal-cycling conditions (Denaturation at 16°C for 30min., Annealing at 42°C for 30min., Extension at 85°C for 5min and hold at 4 °C). Then, Quantitative Real-

time PCR (qPCR) was performed using TaqMan® MicroRNA Assays (20X) which contain a mixture of miRNA-specific forward and reverse primers in addition to TaqMan® probe, under the standard thermal cycling conditions: GoTaq DNA Polymerase activation (1cycle, 2min, 95°C), Denaturation of double-stranded cDNA **(** 40cycle, 15sec, 95°C) then primer annealing and extension (40cycle, 1min, 60°C).

The Ct value of target miRNA was normalized to an endogenous control or reference miRNA (RNU6B) and the expression of miRNA is calculated by the relative quantitative method using the comparative Ct formula (ΔΔCt) [23].

**Statistical Analysis**

Statistical Package for Social Science program (SPSS for Windows, version 24) was used to evaluate the statistical differences by student's t-test, ($P \leq 0.05$) is considered significant and ($P < 0.01$) is highly significant. Results were expressed as mean ± standard deviation (SD). The correlation coefficient (r) between different variables was analyzed by 2-tailed Spearman's correlation. Receiver operating characteristic (ROC) curve analysis (area under the curve (AUC) at 95% confidence intervals (95% CI), sensitivity and specificity) also used to assess the diagnostic performance of each miRNA.

**RESULTS AND DISCUSSION**

**Thyroid Functions and TSHR-Ab**

Forty patients with positive TSHR-Ab were enrolled in this study. Table 1 illustrates mean ± SD of TSH, FT3, and FT4 in the serum of controls and patients. Results in Table 1 showed that levels of FT3 and FT4 in the serum of patients were significantly higher while the TSH level was significantly lower than in the control group ($p < 0.01$). While the levels of FT3, FT4 and TSHR-Ab were non-significantly higher and TSH level was non-significantly lower in patients with goiter and patients with orbitopathy than in patients without goiter and patients without orbitopathy ($p > 0.05$) as shown in Table 2.

**MicroRNAs Expression**

The development in genetic and immunological techniques increases knowledge and gives new insights about the pathogenesis of autoimmune diseases and the possibility to develop new diagnostic and novel therapeutic approaches, therefore miRNAs can be used as biomarkers for various autoimmune diseases [7]. In addition to tissue-derived miRNAs, circulating miRNAs can be used as specific and stable extracellular biomarkers since they are protected from endogenous RNase activity, thus using circulating miRNAs could be minimized the diagnostic approach by a single blood sample [24]. It was reported that results of miRNAs in AITDs have been discordant, with decreased or increased expression, it remains to be determined whether this discrepancy can be attributed to the differences between detection methods used (microarray and/or real time PCR), sample source (thyroid tissue, needle aspiration samples, peripheral blood mononuclear cells (PBMC), and/or serum), or stages of the disease [11].

Figures 1, 2, and 3 show expression of circulating miR-146a-5p, miR-142-3p, and let-7b respectively. Results of the present study showed that expression of the studied miRNAs in serum of patient groups were significantly elevated ($P < 0.01$) compared with healthy controls, indicating that these miRNAs could be used as diagnostic markers, and may provide a new therapeutic approach by targeting miRNAs. These results explain that miRNAs are potential effectors to interrupt the molecular pathways that control the development and function of cells of the immune system by modulating the target gene expression causing an alteration in the normal function of immune cells such as Th17 cell and Tregs [25], leading to loss of immune tolerance in AITD [11], and this may explain the positive correlation of miR-146a-5p ($r = 0.296$, $P = 0.063$) and miR-142-3p ($r = 0.133$, $P = 0.414$) with TSHR-Ab level obtained in this study as shown in Figures 4 and 5.

**Table 1.** Levels of TSH, FT3 and FT4 in the serum of patients and controls

| Subjects (No.) | TSH (μIU/ml) | FT3 (ng/dl) | FT4 (pg/ml) |
|---|---|---|---|
| Controls (40) | 1.092 ± 0.125 | 2.631 ± 0.047 | 1.179 ± 0.0376 |
| Patients (40) | 0.034 ± 0.007 | 5.79 ± 0.639 | 3.209 ± 0.134 |
| P-value | **0.001** | **0.001** | **0.001** |

t-test: patients vs controls ($P < 0.01$ highly significant). Data represent mean ± SD

Another explanation is that miR-146a is involved in immunological tolerance mediated by Tregs, thus alterations in its expression facilitate a pro-inflammatory phenotype of Tregs which characterized by increased production of inflammatory cytokines, such as IFNγ,TNF, IL-17 and IL-2 via overactivation of STAT1 [26]. While miR-142 involve in the pathologic changes of AITD by down-regulating *CLDN1* expression [16].

The negative correlation of let-7b with TSHR-Ab ($r = -0.137$, $P = 0.398$) showed in Figure 6 may be due to that let-7 miRNA sometimes can suppress B-cell activation and reduce the production of antibody [27], or let-7b may contribute to GD development by another mechanism rather than induction of TSHR-Ab production.

**Table 2.** Levels of TSH, FT3, FT4, and TSHR-Ab in serum of patient groups

| Subjects (No.) | TSH (μIU/ml) | FT3 (ng/dl) | FT4 (pg/ml) | TSHR-Ab (U/L) |
|---|---|---|---|---|
| Patients with goiter (32) | 0.037 ± 0.01 | 9.72 ± 2.78 | 3.48 ± 0.336 | 0.785 ± 0.114 |
| Patients without goiter (8) | 0.06 ± 0.027 | 4.81 ± 0.185 | 2.95 ± 0.213 | 0.627 ± 0.119 |
| **P-value** | **0.267** | **0.069** | **0.484** | **0.340** |
| Patients with orbitopathy (30) | 0.036 ± 0.0153 | 7.886± 0.2436 | 1.017 ± 0.268 | 0.694 ± 0.112 |
| Patients without orbitopathy (10) | 0.04 ± 0.0151 | 5.049 ± 0.258 | 0.849 ± 0.321 | 0.621 ± 0.094 |
| **P-value** | **0.426** | **0.279** | **0.808** | **0.650** |

t-test: Patients with goiter vs without goiter (P>0.05 non-significant)
Patients with orbitopathy vs without orbitopathy (P>0.05 non-significant)
Data represent mean ± SD

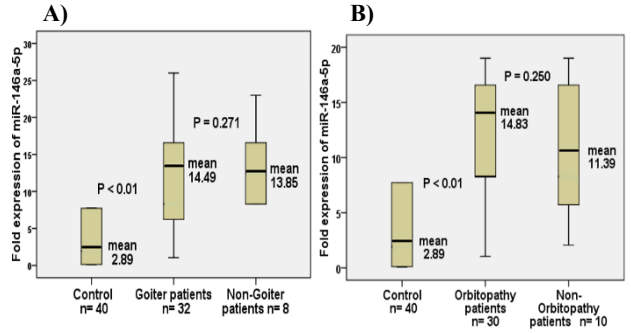


**Figure 1.** Expression of mir-146a-5p in controls and patient groups. **A)** the expression in controls, patients with goiter, and patients without goiter. t-test: controls vs patient with goiter(P<0.01 highly significant), controls vs patient without goiter (P<0.01 highly significant), patients with goiter vs without goiter (P=0.271 non-significant) **B)** Expression in controls, patients with orbitopathy and patients without orbitopathy. t-test: controls vs patients with orbitopathy (P<0.01 highly significant), controls vs patients without orbitopathy (P<0.01 highly significant), patients with orbitopathy vs without orbitopathy (P=0.250 non-significant)

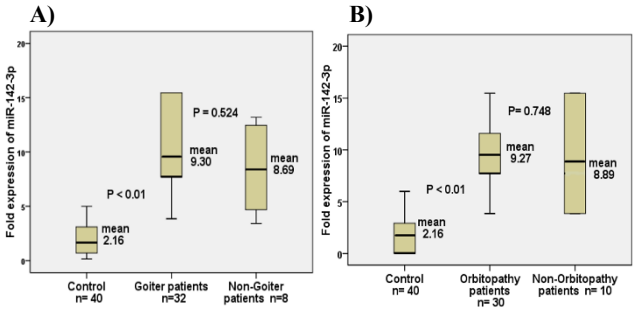


**Figure 2.** Expression of mir-142-3p in controls and patient groups. **A)** The expression in controls, patients with goiter, and patients without goiter. t-test: controls vs patient with goiter (P<0.01 highly significant), controls vs patient without goiter (P<0.01 highly significant) and patients with goiter vs without goiter (p=0.524 non-significant) **B:** The expression in controls, patients with orbitopathy and patients without orbitopathy. t-test: controls vs patients with orbitopathy (p<0.01 highly significant), controls vs patients without orbitopathy (p<0.01 highly significant) and patients with orbitopathy vs without orbitopathy (p=0.748 non-significant)

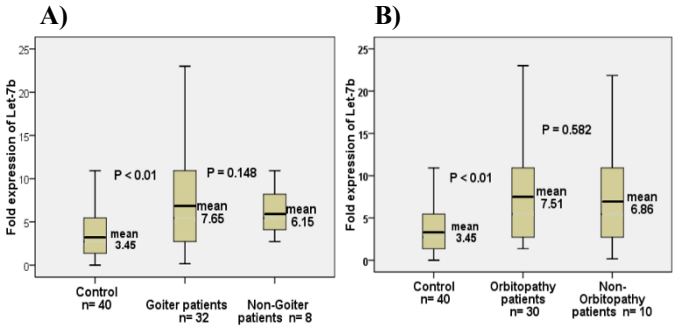


**Figure 3.** The expression of let-7b in controls and patient groups. **A)** the expression of let-7b in controls, patients with goiter, and patients without goiter**.** t-test: controls vs patient with goiter(p<0.01 highly significant)**,** controls vs patient without goiter (p<0.01 highly significant) and patients with goiter vs without goiter (p=0.148 non-significant) **B)** Expression in controls, patients with orbitopathy and patients without orbitopathy**.** t-test: controls vs patients with orbitopathy (p<0.01 highly significant)**,** controls vs patients without orbitopathy (p<0.01 highly significant) and patients with orbitopathy vs without orbitopathy (p=0.582 non-significant)

To identify the contribution of miRNAs in the severity and clinical activity of GD**,** the comparison in miRNAs expression in different patient groups was assessed in this study. Results showed that circulating miR-146a-5p, miR-142-3p and let-7b were non-significantly up-regulated in patients with goiter and patients with orbitopathy (P>0.05) compared with patients without goiter and orbitopathy as shown in Figures 1, 2, 3. This indicated that these miRNAs were not associated with the severity GD. Most miRNAs implicated in the modulation of inflammation in autoimmune disease can exhibit both pro- and anti-inflammatory activities, thus it is not surprising that opposite phenotypes can be observed for the same miRNA depending on its targets [28], therefore some patients involved in the present study showed the severe form of the disease (have goiter and/or orbitopathy) this is due to that miRNAs may have a pro-inflammatory activity causing the appearance of the clinical features while in patients without goiter and orbitopathy these miRNAs may have an anti-inflammatory effect.

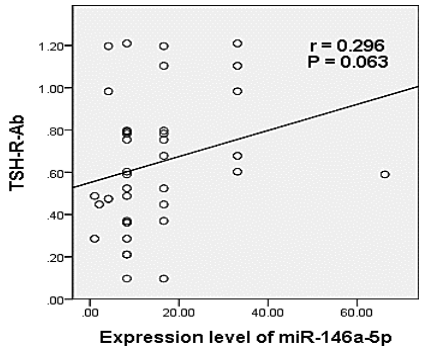


**Figure 4.** Correlation between miR-146a-5p expression (Log fold change) and TSHR-Ab concentration (U/L)

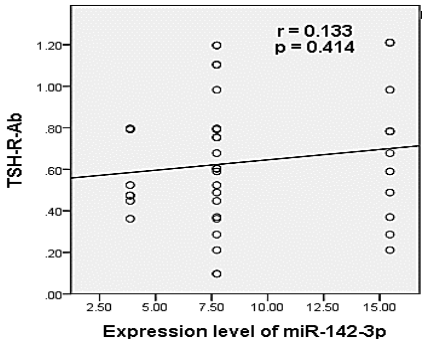


**Figure 5.** Correlation between miR-142-3p expression (Log fold change) and TSHR-Ab concentration (U/L)

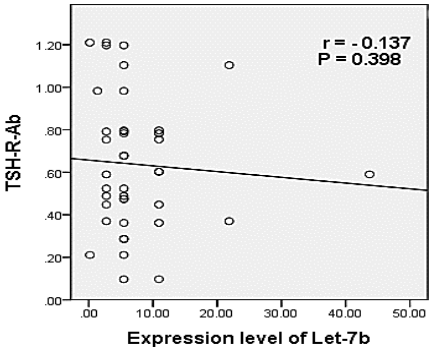


**Figure 6.** Correlation between let-7b expression (Log fold change) and TSHR-Ab concentration (U/L)

Table 3 illustrates the results of ROC curve analyses that showed the discriminating potency of the studied miRNAs. It is found that miR-146a-5p, miR-142-3p, and let-7b have high sensitivity and specificity suggesting that these miRNAs could be used as specific diagnostic biomarkers for GD with good diagnostic accuracy (AUC).

**Table 3.** Receiver operating characteristic (ROC) curve analysis for miRNAs expression

| | **miR-146a-5p** | **let-7b** | **miR-142-3p** |
|---|---|---|---|
| **95% CI** | 0.88 - 0.99 | 0.50 - 0.75 | 0.83 - 0.98 |
| **AUC** | 0.94 | 0.63 | 0.91 |
| **Sensitivity** | 95% | 92% | 82% |
| **Specificity** | 90% | 80% | 80% |

ROC curve analysis: Area under the curve (AUC) at 95% confidence intervals (95% CI), sensitivity and specificity

## CONCLUSION

In conclusion, the up-regulation of miR-146a-5p, miR-142-3p and let-7b in patients and the positive correlation with TSHR-Abs indicating that circulating miRNAs could be used as biomarkers and targets for treatment however there is no association with its clinical features.

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## CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this manuscript.

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