Association of Circulating MicroRNA-142-3p with Graves Disease

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Abstract:
This study aims to investigate the possible role of circulating microRNA-142-3p (miR-142-3p) in the development of graves disease (GD) and its association with the antibody directed against thyroid stimulating hormone receptor (TSHR-Ab) production in patients with GD. Forty patients with positive TSHR-Ab enrolled in this study were divided, based on treatment, into (22 untreated (newly diagnosed) and (18 treated) patients) and based on family history (30 with positive family history and 10 with negative family history). In addition to forty healthy subjects with sex and age matching as a control group. The expression level of circulating miR-142-3p was determined by two steps reverse transcription polymerase chain reaction (RT-PCR) technique. Results show that there is a significant elevation (p < 0.01) in the expression of miR-142-3p in the serum of both treated and untreated patients compared with controls and in patients with positive family history compared with negative family history. While its expression is non-significantly lower (P > 0.05) in the serum of treated patients compared with untreated ones. It has been found that miR-142-3p expression was positively correlated with levels of TSHR-Ab, FT3, and FT4. In addition, the miR-142-3p expression has a good diagnostic accuracy with sensitivity (82%) and specificity (80%). In conclusion, the differential expression of miR-142-3p between patients and healthy controls appears as a potential biomarker for diagnosis of GD and the positive correlation of miR-142-3p with TSHR-Ab suggesting the contribution of this miRNA in the development of GD.

Keywords: Circulating microRNAs, MicroRNA-142-3p, Graves disease, Thyroid stimulating hormone receptor antibody, Reverse transcription polymerase chain reaction

Introduction:
Graves disease is an organ-specific autoimmune disease characterized by diffuse goiter and hyperthyroidism. The diagnosis of GD based on biochemical parameters [free T3 (FT3), free T4 (FT4), thyroid-stimulating hormone (TSH)] and positive thyroid specific autoantibody (TSHR-Ab) (1).

Autoimmune thyroid diseases (AITDs), mainly GD appear as a consequence of loss of immune tolerance toward organ-specific self-antigens, including receptors for TSH. Graves disease is mainly mediated by a humoral autoimmune response, in addition, the T-cell activation is also involved with variable levels of thyroid cell infiltration (2). They induce B-cells to secrete and stimulate TSHR antibody resulting in clinical goiter syndrome, hyperthyroidism and associated orbitopathy and dermopathy (3).

When this autoantibody binds to the TSHR, it activates intracellular G protein ,which in turn, induces transcription of thyroid peroxidase and thyroglobulin genes resulting in thyrocyte hyperplasia and increased thyroid hormone synthesis (4).

Determination of TSHR-Ab level can be used as differential diagnosis for GD before stopping treatment with anti-thyroid drugs (ATDs) (5). In addition, knowledge about the underlying pathogenesis has increased as a result of development in human genetics, molecular immunology, and the presence of murine model for diseases (4).

Understanding the role of circulating miRNAs can provide important and novel information about disease pathogenesis and patient’s clinical condition (6). MiRNA conserved short non-coding RNA, which has a role in regulating the expression of
genes through binding to its target messenger RNAs (mRNAs) at the 3′-untranslated region (3′UTR) resulting in degradation and/or translational inhibition of mRNA (7). In the development of autoimmune disease, miRNAs involved in maintaining tolerance of T and B cells against self-antigens. It also appears to play an important role in the development and differentiation of B cells such as iso-type switching and memory cell response. Thus, alteration in the expression of miRNAs is associated with the appearance and monitoring several autoimmune diseases (8). MicroRNAs can be secreted into the bloodstream defined as circulating miRNAs and it can be used as a prognostic, diagnostic as well as a therapeutic target for a variety of diseases (9) and (10).

The aim of this study is to determine the alteration in the expression of circulating miR-142-3p between control group and patients with GD, and to evaluate the possible association of this miRNA with TSHR-Ab in GD patients.

Materials Methods:
Collecting samples

Blood samples were collected from forty patients (9 males and 31 females) with age range of (25 – 50 year) complaining from GD during the period from May 2019 to November 2019 attending Baghdad Center for Radiotherapy and Nuclear Medicine. Clinical diagnosis of patients was confirmed by specialist based on clinical features (goiter and/or orbitopathy), hormonal study (FT3, FT4 and TSH) and positive TSHR-Ab. The forty patients were divided based on treatment state into 22 untreated (newly diagnosed) and 18 treated patients and based on family history (30 with positive family history and 10 with negative family history). In addition to forty healthy subjects with sex and age matching, having normal levels of FT3, FT4 and TSH as control group. All patients and controls are free from any other autoimmune diseases. Individuals enrolled in this study have been asked to give blood and information. The ethical approval of this study was obtained from the University of Baghdad- College of Medicine and Health Ministry in Iraq.

Measurement of thyroid functions and TSHR-Ab

The measurement of TSH, FT3 and FT4 were done by using kits supplied from TOSOH Bioscience/Japan performed on Automated Florescent Enzyme immunoassay System (TOSOH AIA-360). The normal range of TSH concentration is (0.38 – 4.31 μIU/ml), FT3 concentration (2.17 - 3.34 pg/ml) and FT4 concentration (0.82 – 1.63 ng/dl).

Thyroid stimulating hormone receptor autoantibody concentration was determined with indirect enzyme-linked immuno-sorbent assay (ELISA) using TSHR-Ab ELISA Kit supplied by Demeditec/Germany. TSHR-Ab concentration of (>1.5 U/L) is considered as positive which is equivalent to the Absorbance of (< 1.2).

Determining the expression level of miR-142-3p by two steps RT-PCR

MicroRNA was extracted from serum samples using Trizol reagent supplied by Ambion Life Technologies, USA. Serum sample was homogenized by the addition of 1ml of Trizol reagent to 0.4 ml serum then stored at -20 °C until used. Determination of RNA concentration and purity for each sample was done by measuring the absorbance at 260nm (A260) and 280nm (A280) for by Nanodrop spectrophotometer (BioDrop, England). Then reverse transcription was performed to convert the extracted miRNA of each sample into cDNA with high specificity using TaqMan MicroRNA reverse transcription kit and reverse transcription primer specific for each miRNA supplied by Applied Biosystems/Thermo fisher scientific, USA. Reverse transcription was carried under thermal cycling conditions (Denaturation 16°C (30min.), annealing 42°C (30min. ) , extension 85°C (5min) ; hold 4 °C). Determination of miR-142-3p expression was done by Quantitative real time PCR (qPCR) using TaqMan® MicroRNA Assays (20X) containing mixture of miRNA-specific forward and reverse PCR primer in addition to TaqMan® MGB probe. Real time PCR was done under the following conditions:

- GoTaq DNA Polymerase activation 1 cycle for 2 min at 95 °C, Denaturation of double-stranded cDNA 40 cycle for 15 s at 95 °C then Primer annealing and extension 40 cycle for 1 min at 60 °C.

The expression of miRNA is determined by relative quantitative method in which the fold changes in the expression level of patient’s miRNAs to the healthy control is calculated using the comparative Ct formula (ΔΔCt). The Ct value of target miRNAs is normalized to an endogenous control (reference miRNA) (RNU6B has been used as reference gene in this study) and relative to a healthy control (11).

Statistical analysis

Statistical Package for Social Science program (IBM SPSS for Windows, version 24) has been used. Mean ± SD is used to express the results. The statistical difference is estimated using student’s t test, P ≤ 0.05 is significant and (P<0.01) is highly significant. Correlation coefficient (r) between different variables has been analyzed by 2-tailed Spearman’s correlation. Receiver operating
characteristic (ROC) curve analysis (AUC at 95% confidence intervals, sensitivity and specificity) has been used to distinguish between patients and controls and to assess the diagnostic performance of miRNA.

Results and Discussion:
Thyroid functions and TSHR-Ab

It has been found that the levels of FT3 and FT4 in serum of patients are significantly higher (p < 0.01) than in serum of healthy controls. While the level of TSH in patient’s serum is significantly low (p< 0.01) compared with its level in serum of controls as shown in (Table 1). This is in accordance with the previous report which stated that GD is diagnosed biochemically by elevated serum FT3 and/or FT4 and low TSH level (12). It is important to note that GD is caused by TSHR-Ab which binds to the TSHR on the surface of thyrocytes. Then abnormally activate thyroid gland, resulting in uncontrolled overproduction of thyroid hormones causing hyperthyroidism (13,14,15).

Table 1. Levels of TSH, FT3 and FT4 in patients with GD and control groups

| Subjects | TSH(μIU/ml) | FT3(ng/dl) | FT4(pg/ml) |
|----------|-------------|------------|------------|
| patients | 0.034 ± 0.007** | 5.79 ± 0.639** | 3.209 ± 0.134** |
| control  | 1.092 ± 0.125 | 2.631 ± 0.047 | 1.179 ± 0.0376 |
| P-value  | 0.001 | 0.001 | 0.001 |

Table 2. Levels of TSH, FT3, FT4 and TSHR-Ab in treated and untreated patients

| Variable | Untreated patients (n=22) | Treated patients (n=18) | p-value |
|----------|---------------------------|-------------------------|---------|
| TSH(μIU/ml) | 0.055 ± 0.017 | 0.362 ± 0.099 | 0.133 |
| FT3(ng/dl)  | 7.032 ± 1.344 | 4.822 ± 0.909 | 0.123 |
| FT4(pg/ml)  | 3.209 ± 0.161 | 3.035 ± 0.198 | 0.180 |
| TSHR-Ab(U/L) | 0.739 ± 0.056 | 0.531 ± 0.076 | 0.050 |

The concentrations of free form of T4 and T3 (FT4 and FT3) have been measured in this study, since the total forms have been affected by thyroid hormone-binding proteins while measuring the FT4 and FT3 has been less affected, thus it is useful for differential diagnosis of destructive thyroiditis and GD (16). It has been found that; FT3 and FT4 levels in serum of treated patients showed non-significant decrease (P > 0.05) compared with untreated patients and TSH level in serum of treated patients is non-significantly higher (P > 0.05) than its level in untreated patients. Regarding to TSHR-Ab, it is found that TSHR-Ab level in serum of treated patients is significantly lower than its level in untreated patients as shown in (Table 2).

This agrees with the study that found TSHR-Ab level declined with ATD therapy and after thyroidectomy (15). In addition, this was in agreement with other studies that found higher levels of FT3, FT4 and TSHR-Ab associated with the lower TSH ones in untreated GD patients compared with treated patients (17) and (6).

The important reason for measurement of TSHR-Ab is for GD diagnosis, monitoring disease activity, treatment choices and as biomarker in predicting GD relapse and remissions. However, TSHR-Ab can predict short-term hyperthyroidism relapse after a course of antithyroid medications but it is less effective in predicting long-term relapses or remissions (15). Thus, it is important to find clinical and molecular biomarkers for monitoring disease activity and to increase understanding the pathogenic mechanisms of autoimmune thyroid diseases to develop good treatment choice. Thus, in this study measurement of miR-142-3p expression in both treated and untreated patients in addition to association between miR-142-3p and TSHR-Ab were determined.

Correlation of miR-142-3p expression level with TSHR-Ab

As a common organ-specific autoimmune disease, GD is characterized by diffuse goiter and hyperthyroidism. The diagnosis of GD is based on biochemical parameters (FT3, FT4, TSH) and positive thyroid specific autoantibody TSHR-Ab (1). However, the development in genetic and immunological techniques increases knowledge and gives new insights about the genesis of autoimmune diseases and the possibility to develop new diagnostic and novel therapeutic approaches (18). Therefore, miRNAs can be used as biomarkers for various autoimmune diseases (19). Although there are many studies that have investigated the association of miRNAs in thyroid tissue of patients withAITD, few studies focused on their clinical and diagnostic utility and their role in development of GD (20). In addition, in Iraq there are no previous studies or information about the role of circulating miRNAs in GD. Thus, the present study is designed to detect the expression level of miR-
142-3p and its association with the pathogenesis of GD and to assign a risk for the development of AITDs which may help in selecting appropriate therapeutic strategies by targeting these miRNAs. Studies on miRNAs in AITDs have been discordant, with decreased or increased expression of miR-142-3p. 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 (PBMCs), and/or serum), or stages of the disease development and may be depending on the mechanism of these miRNAs in modulating the immune response of diseases.

MicroRNAs play an important role in the pathogenesis of autoimmune diseases mainly due to their modulatory effects in the immune system (21). Disrupted expression of miRNAs is related to different autoimmune diseases especially those involved in Th17/T-regulatory (Tregs) cells balance (22). miR-142 has a significant attention due to the essential role in immune responses regulation; it has a crucial role in hematopoietic cells lineage differentiation (23). Thus miR-142 can be used for diagnosis of various autoimmune diseases. The level of miR-142-3p was detected for both patient and control groups. Results found that there is a significant elevation (p < 0.01) in the expression of miR-142-3p in serum of both treated and untreated patients compared with controls (Fig.1) and in patients with positive family history compared with negative family history (Fig. 2) indicating that this miRNA may have a crucial role in GD development and its overexpression can play an important role in increasing the risk for development of AITDs, thus the level of circulating miR-142-3p could be used as a helpful marker to identify people at familial risk for developing GD.

This may be due to role of miR-142-3p in regulating immune response; it involves in differentiation process of macrophage and dendritic cells, in addition to phagocytosis by myeloid inflammatory cells. Therefore, miR-142-3p may be associated with the secretion of different inflammatory factors (24). This agrees with a study that found that miR-142-3p level was up-regulated in thyroid tissue of GD patients (20) and with another study that found that overexpression of miR-142-3p made dendritic cells producing increased level of IL-6 that inhibits Tregs function and induce it to release more IL-17 and less IL-10, thus breaking the balance between Th17 and Treg which is an important cause for development of autoimmune response (25).

Based on treatment, it has been shown that level of miR-142-3p slightly decreased (P > 0.05) in serum of treated compared with untreated patients (Fig.1), suggested that miR-142-3p could be used as biomarker to anti-thyroid drug response. This result was in consistence with a previous study which found that the level of circulating miR-142-3p increased in untreated GD patients, and gradually decreased in patients under anti-thyroid treatment (17). Also, another study stated that alteration in miRNAs expression may reflect the response to therapy (6). In addition, a study found that up-regulated level of miR-142 was decreased in autoimmune arthritis patients after celestrol treatment (26).

To assess the existence of any possible role of miR-142-3p in the pathogenesis of GD, the correlation of its expression with patients’ corresponding serum levels of TSHR-Ab, FT3, FT4, and TSH was investigated. Results of correlation analysis showed the miR-142-3p has a positive correlation with TSHR-Ab, FT3, and FT4, while it is negatively correlated with TSH level as shown in (Fig.3). This suggests that the main mechanism by which miR-142-3p contributes to GD pathogenesis involves the production of TSHR-Ab which in turn causes the production of FT3 and FT4. This result is in agreement with a study that reported that some miRNAs are positively correlated with TSHR-Ab and associated with the development of GD (27). The positive correlation of miR-142-3p with TSHR-Ab may be due to that miR-142-3p was involved in the inflammatory pathways such as B-cell receptor signaling pathway and TNF-signaling pathway, which is responsible for the production of antibody and in turn resulting in its positive correlation with FT3 and FT4. On the contrary, another study found that there was no correlation between miR-142-3p and thyroid function parameters (17).

Another explanation of this result is that miR-142 in AITDs 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 (28). This is in consistence with a study suggesting that miR-142 involved in the pathologic changes of AITDs by down-regulating CLDN1 expression (29). The discriminating power of miR-142-3p between GD patients and control group was estimated using ROC curve analyses (Table 3). The results show that the level of miR-142-3p has a good diagnostic accuracy AUC value (0.91) at 95% CI (0.83 - 0.98)
suggesting that this miRNA can be used as specific biomarker in GD diagnosis with high sensitivity (82%) and specificity (80%).

In conclusion, the differential expression of miR-142-3p between patients and healthy controls can be expressed as potential biomarker for diagnosis of GD and the positive correlation of miR-142-3p with TSHR-Ab level suggesting the contribution of this miRNA in the development of GD.

![Figure 1. Expression of miR-142-3p in controls and patient groups](image)

**Figure 1.** Expression of miR-142-3p in controls and patient groups

Controls vs untreated patients; p < 0.01 (highly significant)
Controls vs treated patients; p < 0.01 (highly significant)
Treated vs untreated patients; p >0.05 (non-significant)

![Figure 2. Expression of miR-142-3p in patients with positive and negative family history](image)

**Figure 2.** Expression of miR-142-3p in patients with positive and negative family history

T-test; patients with positive vs negative family history (**P < 0.01 highly significant)

![Figure 3. Correlation of miR-142-3p expression with thyroid functions and antibody level. a. Correlation of miR-142-3p with TSHR-Ab, b. Correlation of miR-142-3p with TSH, c. Correlation of miR-142-3p with FT3, d. Correlation of miR-142-3p with FT4](image)

**Figure 3.** Correlation of miR-142-3p expression with thyroid functions and antibody level.

a. Correlation of miR-142-3p with TSHR-Ab, b. Correlation of miR-142-3p with TSH, c. Correlation of miR-142-3p with FT3, d. Correlation of miR-142-3p with FT4

(Correlation is significant *p ≤ 0.05)
Table 3. ROC curve analysis for miR-142-3p expression

|          | miR-142-3p |
|----------|------------|
| 95% CI   | 0.83 - 0.98|
| AUC      | 0.91       |
| Sensitivity | 82%       |
| Specificity | 80%        |

AUC: area under the curve, 95% CI: 95% confidence intervals

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Authors’ declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Al-Ma’moon

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علاقة الحمض النووي الرايبي الدقيق (miR-142-3p) مع مرض اعتلال الغدة الدرقية المفرط (Graves disease)

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الخلاصة:
تهدف هذه الدراسة إلى تحديد وجود دور محتمل للحمض النووي الرايبي الدقيق (miR-142-3p) في ظهور مرض اعتلال الغدة الدرقية المفرط (Graves disease) وعلاقته مع افراز الأجسام المضادة لمستقبلات الهرمون المحفز للدرقية (TSHR-Ab). تم قياس مستوى تعبير (miR-142-3p) في مصل دم 40 مريضاً يعانون من مرض اعتلال الغدة الدرقية المفرط ويشملون 22 مريضاً دون علاج و18 مريضاً يأخذون علاج بالإضافة إلى اربعين شخصاً كمجموعة ضابطة. جميع المرضى والاصحاب لا يعانون من أي مرض مناعي ذاتي. قام بالاستخدام تقنية RT-PCR. أظهرت النتائج أن هناك زيادة محسنة (p < 0.01) في تعبير (miR-142-3p) في المرضى المصابين بالمرض في مقارنة مع المجموعة الضابطة. هناك علاقة موجبة بين مستوى تعبير (miR-142-3p) وتركيز الأجسام المضادة للهرمون المحفز للدرقية (TSHR-Ab) بالإضافة إلى أن هذه العلاقة موجبة بين مستوى تعبير (miR-142-3p) وتركيز الأجسام المضادة للهرمون المحفز للدرقية (TSHR-Ab) وتركيز الأجسام المضادة للهرمون المحفز للدرقية (TSHR-Ab). نستنتج من هذه الدراسة أن الخلل في تعبير miR-142-3p يمكن أن يكون مستقبل للعديد مناضات الفيروسا في ظهور مرض اعتلال الغدة الدرقية المفرط. النتائج تعزز من الأهمية التشخيصية للحمض النووي الرايبي الدقيق (miR-142-3p) في مرض اعتلال الغدة الدرقية المفرط وتشير إلى أن هناك فرصة ملحة لاستخدام الحمض النووي الرايبي الدقيق (miR-142-3p) كمرشح جيد ل التشخيص المبكر للمريض المصاب بمرض اعتلال الغدة الدرقية المفرط.

الكلمات المفتاحية: الحمض النووي الرايبي الدقيق، الحمض النووي الرايبي الدقيق (miR-142-3p)، مرض اعتلال الغدة الدرقية المفرط، تفاعل البلمرة المتسلسل العكسي (miR-142-3p).