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Study the Role of Mirna146a and Some Interleukins as Important Markers in Patients with a Thyroid Disorder

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Abstract. MicroRNAs (miRNAs) are a class of non-coding RNAs approximately 19–24 nucleotides in length that can function as oncogenes or tumour suppressors by inhibiting the translation of tumour suppressor genes or by blocking the translation of oncogenes. 120 samples were collected from the governorate of Najaf (60 with thyroidectomy and 60 as controls) to identify Using miRNA146a as an indicator for primary of thyroid cancer. The proportion of patients was over the expiration of miRNA146a as (2.32±0.12) compared to the control group (1±0.07)),. This study also by using the ELISA technique for interleukin 6 and tumor necrotic factor alpha (TNFα) as markers for thyroid disorder This study show the high significance of interleukin 6 (IL6) and tumor necrotic factor alpha (TNFα) as (p-value 0.002) and (p-0.004) respectively.
Keywords: miRNA146a, interleukin 6, and tumor necrotic factor alpha (TNFα)

1 Introduction

Thyroid cancer is the most common endocrine malignancy with the papillary variant being the most common. In recent years there has been an increase in the incidence of papillary thyroid cancer. Tyroid cancers can be classified as three types: well-differentiated, poorly differentiated and anaplastic carcinomas[1] . Papillary thyroid carcinoma (PTC) is a well-differentiated type, accounting for 70–80% of thyroid carcinomas [2] miRNAs regulate proliferation, metastasis and apoptosis by inhibiting tumor suppressor gene pathways or activating oncogenic pathways[3]. Almost half of human miRNAs are in the position of fragile sites and genomic regions that are associated with cancers[4]. The epigenetic profile of TC, namely alterations in microRNA (miRNAs) expression, has been determined to modulate gene expression [5]. Genome-wide analyses have generated specific miRNA profiles of different histotypes of TCs and identified the upregulated and downregulated miRNAs related to various carcinogenesis stages and prognoses.[6]

Interleukin-6 (IL-6), is a pleiotropic cytokine that acts as both a pro-inflammatory and anti-inflammatory activity[7]. It has a profound effect on B cells, promoting plasma cell differentiation and immunoglobulin (Ig) secretion, and also enhances T cell proliferation[8]. Recent evidence has suggested that IL-6 may play important roles in the development of specific immune response, as described mice lacking IL-6 or TNF-α gene expression are deficient in T and B cell function, IL-6 drives differentiation of activated naive Th cells into IL-17 and IL- 2 expressing Th17 and Th22 cells, key for anti-bacterial and anti-fungal defense. Conversely, IL-6 inhibits differentiation of CD4+ T regu-latory cells, which play a key role in restraining inflammatory responses.[9].

tumour necrosis factor alpha (TNFα) as an pro-inflammatory mediatorshas been identified and it is an important player in cancer progression and metastasisTumor [10] necrosis factor alpha (TNF-α_) is a cytokine that has pleiotropic effects on various cell types. It has been identified as a major regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and autoimmune diseases[11] It is functionally known to trigger a series of various inflammatory molecules, including other cytokines and chemokines. TNF-_ exists in a soluble and transmembrane form. The transmembrane TNF-_ (tmTNF-_) is the initially synthesized precursor form and is required to be processed by TNF-_-converting enzyme (TACE), a membrane-bound disinterring metalloproteinase, to be released as the soluble TNF-α_ (sTNF-α) .[12] .

Methods

2: Ethical Consideration

After the protocol was authorised by the Ethical Review Board for human studies at the Faculty of Nursing/University of Kufa/Iraq (No. 10-04-01/2015), all subjects submitted theirwritten informed consent prior to enrollment.

3: Individuals

The study was a case-control study conducted on 120 individuals between February and September of 2022, dividing them into two groups: 60 individuals who underwent a thyroidectomy and 60 individuals who appeared to be healthy [. 5 ml of blood were collected from the individual, and the epidermis around the arm was sterilised with 70% ethyl alcohol before the blood was divided into two portions as follows: The first portion (2 ml) from both groups was transferred into anticoagulant tubes and promptly frozen at -20 degrees Celsius for use in a molecular study. After allowing the blood to coagulate for 30 minutes at room temperature, the remaining volume (3 ml) was transferred into a gel tube for serum separation. then centrifugated the blood for 5 minutes at 4000 rpm. After that, the serum wasgathered in five sterile Appendrofe tubes and kept at -20 degrees Celsius to determine the level of interleukin 6(IL6) and tumour negrosis factor alpha (TNFα)by Enzyme Linked Immunosorbent assay (ELISA).

4: Procedure

4.1: miRNA146a:

Samples of whole blood were collected from the patient group and healthy control group in EDTA tubes. Then by using TransZol , RNA was extracted from whole blood.

Principle

TransZol lyses cells with guanidine isothiocyanate. In the process of sample lysis, TransZol can maintain the integrity of RNA. After adding RNA Extraction Agent, the solution is divided into a colourless aqueous phase and a pink organic phase. RNA is in the aqueous phase. RNA can be recovered by precipitation with isopropanol. Isopropyl alcohol recovers protein. Suitable for rapid extraction of total RNA from a variety of tissues and cells.

Steps of RNA extraction :

The Genomic RNA from blood samples were extracted by using a Favorgen RNA extraction kit (Whole blood Favorgen, korea), and done according to company instruction as following steps:

1: Transfered suspension cells to a microcentrifuge tube. Centrifuge the ample at 8,000× g for 2 minutes at 2-8°C, discard the supernatant. Added 1 ml of TransZol per 10' cell, pipetting up and down until no visible precipitates are present in lysa, Incubate at room temperature for 5 minutes.

2: .Added 0.2 ml of RNA Extraction Agent per ml TransZol used. Shake the tube vigorously by hand for 15 seconds. Incubate at room temperature for 3 minutes.

3: .Centrifugated the sample at 10,000×g for 15 minutes at 2-8°C. The mixture separates into a lower pink organic phase, interphase, and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase was around 60% volume of TransZol reagent.

4: Transfered the colorless, upper phase containing the RNA to a fresh RNase-free tube. Added 0.5 ml of isopropanol for per ml TransZol used. Mix thoroughly by inverting the tube. Incubated at room temperature for 10 minutes.

5: .Centrifugated the sample at 10,000×g for 10 minutes at 2-8°C. Discard the supernatant. Colloidal precipitate can be seen at the wall and the bottom of the tube.

6:Added 1 ml of 75% ethanol (prepared with RNase-free water), vortexing vigorously (added at least 1 ml of 75% ethanol for 1 ml TransZol used)

7: Centrifugated the sample at 7,500×g for 5 minutes at 2-8°C.

8: Discard the supernatant. Air-dry the RNA pellet (about 5 minutes).

9: RNA pellet is dissolved in 50-100 µl of dissolving solution.

10: Incubated at 55-60°C for 10 minutes. For long-term storage, store the purified RNA at -70°C.

4.2: Estimation of total RNA concentration and purity

The purity of samples was measured by UV/Visable spectrophotometer instrument by adding extracted RNA in the instrument. A260/280 ratios of pure RNA would usually at 2.0.

4.3. Reference Gene Selection

The reference gene or housekeeping gene or endogenous control gene selected by finding the best and the most stable reference gene expressed in serum samples. The best reference gene depending on three parameters first, high expression level. Second, stable and expressed among all sample. Third, show the Converge expression level among all samples[13].

4.4. Determination of miR-146a and U6 reference gene Expression in Samples by one step RT-qPCR

GoTaq 1-Step RT-qPCR System combines GoScrip Reverse Transcriptase and GoTaq qPCR Master Mix in a single-step real-time amplification reaction. The system, which optimized for RT-qPCR, contains a proprietary fluorescent DNA binding dye, Sybr Green Dye. The system enables the detection of RNA expression levels using a one-step RT-qPCR method:

- GoTaq® 1-Step RT-qPCR component, total RNA, primers and Nuclease-free water all thawed on ice and each solution mixed well.
- GoTaq® 1-Step RT-qPCR reaction was prepared as shown in Table (1).

RT-qPCR reactions performed using the cycling program shown in Table (2).

Table (1). : GoTaq® 1-Step RT-qPCR Reaction Mix.

Component	Volume Final	Concentration
GoTaq® qPCR Master Mix, 2X	10 µl	1X
GoScript™ RT Mix for 1-Step RT-qPCR (50X)	0.4 µl	1X
Forward Primer (20X)	2 µl	300 nM
Reverse Primer (20X)	2 µl	300 nM
MgCl2	1.6 µl	≥2 mM
RNA template	3.7 µl	100 ng
CXR Reference Dye	0.3 µl	≥33nM

Table (2): One step RT-qPCR programs.

Step	Temperature	Duration	Cycles
Reverse transcription	37 C̊	15 min	1
RT inactivation/Hot-start activation	95 C̊	10 min	1
Denaturation	95 C̊	10 sec	50
Annealing	58 C̊	30 sec	
Extension and data collection	72 C̊	30 sec	

4.5: primer of miRNA146a

The primers (table 3), which are used in qPCR for the detection of miR-146a [14] and reference gene U6[15] were provided by Macrogen company (Korea).

Table (3): The sequence of primers that used in the present study.

Primer	Direction	Sequence	Reference
miR-146a	Forward	TGAGAACTGAATTCCATGGGT	[14]
	Reverse	GCAGGGTCCGAGGTATTC	
U6	Forward	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	[15]
	Reverse	CTCAACCTACAATCAAAAACAACACAAACA	

4.6: Calculating Gene Expression (Gene Fold)

2 Gene expression or gene fold or RQ (Relative quantification) value calculated by Pfaffl equation

$$RQ = 2^{-(\Delta\Delta CT)}$$ **3**

4 Gene fold is calculated firstly by collecting CT (CT - cycle threshold) average value from real time PCR device for each triplicated sample then calculate ΔCT value for each sample as follow:

5 Δ CT = CT (gene of interest) – CT (reference gene)

6 ΔCT is the difference in CT values for the gene of interest and reference gene for a given sample. This is essential to normalize the gene of interest to a gene, which not affected by the experiment.

7 To calculate ΔΔCT value which found as follows:

8 ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control))

9 After calculating ΔΔ CT for all samples then take final equation to calculate gene expression (fold change) as follows:

10 Fold gene expression RQ = 2-(ΔΔCT)

2.3.2: The Enzyme-Linked Immunosorbent Assay (ELISA)

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to IL6 . and TNF-α. Standards or samples are added to the appropriate Microelisa stripplate wells and combined with the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for TNF-α is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain TNF-α and HRP conjugated TNF-α antibodies will appear blue and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of TNF-α. You can calculate the concentration of TNF-α in the samples by comparing the OD of the samples to the standard curve.

A. Preparations Prior to Pipetting: "According to the Manufacturing Instructions".

1: Wash Buffer 30X: Distal water was used to dilution wash buffer by adding 20 ml from buffer to 580 ml water for IL6 .and TNF-a.

2: Dilution of stander: to make series of standers from stock stander depending of the type of elize kit.

Procedure:

1. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µlSample dilution buffer and 10µl sample were added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.
2. Incubation: incubate for 30 min at 37℃ after sealed with Closure plate membrane.
3. Washing: carefully peel off Closure plate membrane, aspirate and fill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

4. Added 50 µl HRP-Conjugate reagent to each well except the blank control well.
5. Incubation for 30 min at 37℃ after sealed with Closure plate membrane.
6. Washing carefully peel off the Closure plate membrane, aspirate and fill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times..
7. Colouring: Added 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubated at 37 ℃ for 15 minutes. Please avoid light during coloring.
8. Termination: Add 50 µl stop solution to each well to terminate the reaction. The colour in the well should change from blue to yellow.
9. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. The assay should be carried out within 15 minutes after adding the stop solution.

Calculation of the Results

For the three variables interpretation of the results has established the standard curve by plotting the optical density (OD) of each standard (y-axis) with respect to the corresponding concentration values in pg/ml (x-axis).

5: Statically Analysis

Statistical information was documented as mean standard error (SE). The Graph Pad Prism version 7 program was used to conduct a statistical test. The statistical significance threshold was set at a P-value of 0.05 .

6: Results

6.1 Detection of miRNA146a in patients with thyroid disorder:

In the current study, it was detected miRNA as a precursor to the occurrence of thyroid cancer, the result of the study by using the RT-PCR technique has been shown high expression in miRNA146a in a patient with thyroid disorder as SD (2.32±0.12) compared to control with SD (1±0.07) (p-0.001) . Figures (1) and (2).

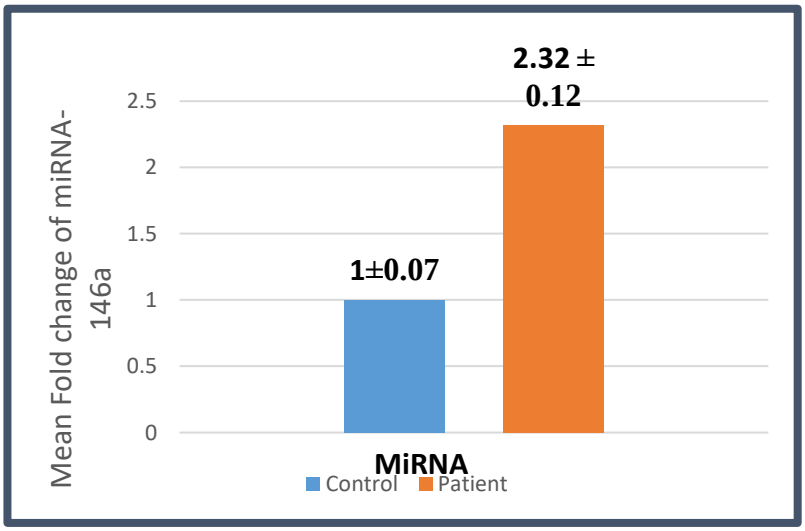

Figure (1) Detection of miRNA146a in patient with thyroid disorder:

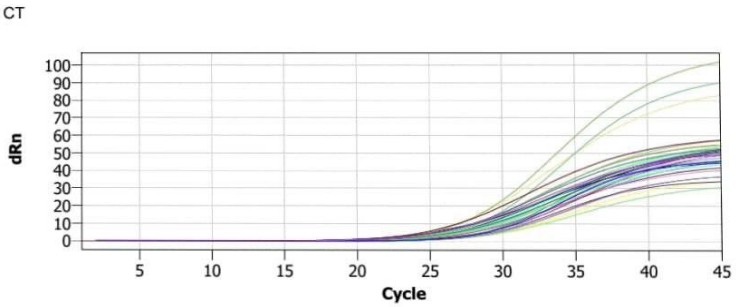

Figure (2) Real-time PCR image show the Ct value of selected microRNA genes

6.2 : The connection between IL6 and miRNA146a in all patients with and without EBV and controls:

This result of IL6 show high significance (p-0.002) for positive and negative EBV with Mean ± SE (6.07± 0.98) , (5.5 ± 0.36) respectively compared with (4.48 ± 0.18) for control and between miRNA with IL6 control in Mean ± SE(2.32 ± 0.7), (4.48 ± 0.18) respectively.Table (4).

Table (4) The association between IL6 and miRNA146a in all patients with and without EBV and controls

MiRNA Mean ± SE	IL6			P-value 0.002
	EBV (Positive) Mean ± SE	EBV (Negative) Mean ± SE	Control Mean ± SE	
2.32* ± 0.7	.07± 0.98	5.5 ± 0.36	4.48 ± 0.18	

6.3 : The connection between TNFα and miRNA146a in all patients with and without EBV and controls:

This result of TNFα show high significant (p-0.004) for positive and negative EBV with Mean ± SE (20.09± 5.06) , (19.5 ± 1.92) respectively compared with (13.32±1.36) for control and between miRNA with IL10 control in Mean ± SE(2.32 ± 0.7), (13.32±1.36) respectively (Table (5)

Table (5) The association between miRNA146a and TNFα in all patients and controls

MiRNA Mean ± SE	TNFα			P-value
	EBV (Positive) Mean ± SE	EBV (Negative) Mean ± SE	Control Mean ± SE	
2.32* ± 0.7	20.09± 5.06	19.5 ± 1.92	13.32±1.36	0.004

7: Discussion

MiR-146a is unique in that it is significantly enriched in human tissues of the skin, cervical, breast, pancreas and prostate cancers compared to the same noncancerous tissues.[16]

This study agreement with Wang etal.,(2017) who found a significantly elevated expression of miRNA-146a in the plasma of patients with Graves' disease (n = 27) compared to controls (n = 15).Also, This study concurred with a study of [18] that elevated miR-146a levels in PTC (papillary thyroid carcinoma) , cervical cancer, breast cancer and pancreatic cancer,whereas reduced miR-146a expression is associated with prostate cancer, showed that miR146 was significantly overexpressed in PTC with >1.5-fold changes in at least seven patients. Gene variation of miR-146a affected the expression of mature miRNAs and was linked to the risk of PTC . [19] . expression of miRNA146a was significantly higher in patients before treatment compared with their levels both in healthy subjects and after treatment samples.[20] .

This study agreement with the study of Motedayyen et al which showed the miRNA146a significant reduction in the expression level of TNF-a and IL-6 in patients compared with the control group (P <0.001) [21]. Also,

Zhou et al., proposed the regulation of IL-6 expression by miRNA-146-a in sepsis because the down-regulation of miR-146a caused an elevated expression of IL-6, and might be responsible for the stimulation of monocyte proliferation., [22]. Furthermore, Suppression of miRNA-146-a increased the expression levels of IL-6, TNF-α, intercellular adhesion molecule-1 and E-selectin with increased Nuclear factor kappa B NF-κB activity and thus miRNA-146-a could be a possible therapeutic agent for sepsis. [22]

Also, this study concurred with a study of[23] that miR-146a is involved in immunological tolerance mediated by Tregulatory cells (Tregs), thus alterations in miR-146a 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 over activation of signal transducer and activator of transcription-1 (STAT1) which is a direct target of miR-146a[23]. Béres et al.findings on human biopsies, that TNF-a treatment increases the expression of miR-146a and -155, but not miR-122 in HT-29 colonic epithelial cells[24]. Also, Çarman et al., found that the levels of TNF-α and expression of miRNA 146a were significantly increased in febrile seizure (FS) patients. higher in the group than in the control group, ($p<0.05$) .[25]. the levels of IL-6 and TNF-α in the peripheral blood of patients with CAS stenosis were higher than those in the normal group. [26].

8: Conclusion

Our findings revealed that miRNA146a is one of primary indicators of infection, of thyroid cancer and it was also discovered thatIL6 and TNFα were pro-inflammatory cytokines for thyroid disorder.

9: References

[1] J.-Y. Scoazec and A. Couvelard, "Classification of pancreatic neuroendocrine tumours: changes made in the 2017 WHO classification of tumours of endocrine organs and perspectives for the future," in *Annales de pathologie*, 2017, vol. 37, no. 6, pp. 444–456.

[2] K. R. Joseph, S. Edirimanne, and G. D. Eslick, "Thyroidectomy for thyroid cancer in the elderly: A meta-analysis," *Eur. J. Surg. Oncol.*, vol. 45, no. 3, pp. 310–317, 2019.

[3] B. Liao *et al.*, "Long noncoding RNA CTC inhibits proliferation and invasion by targeting miR-146 to regulate KIT in papillary thyroid carcinoma," *Sci. Rep.*, vol. 10, no. 1, p. 4616, 2020.

[4] Y. Feng, Y. Zhang, D. Zhou, G. Chen, and N. Li, "MicroRNAs, intestinal inflammatory and tumor," *Bioorg. Med. Chem. Lett.*, vol. 29, no. 16, pp. 2051–2058, 2019.

[5] W. Tan, B. Liu, S. Qu, G. Liang, W. Luo, and C. Gong, "MicroRNAs and cancer: Key paradigms in molecular therapy," *Oncol. Lett.*, vol. 15, no. 3, pp. 2735–2742, 2018.

[6] C. A. Silaghi *et al.*, "The prognostic value of MicroRNAs in thyroid cancers—a systematic review and meta-analysis," *Cancers (Basel).*, vol. 12, no. 9, p. 2608, 2020.

[7] H. T. Nanakaly, "Interleukine-6 level in saliva of patients with chronic periodontitis: A case-control study," *J. baghdad Coll. Dent.*, vol. 325, no. 3154, pp. 1–6, 2016.

[8] Y.-D. Xu, M. Cheng, P.-P. Shang, and Y.-Q. Yang, "Role of IL-6 in dendritic cell functions," *J. Leukoc. Biol.*, vol. 111, no. 3, pp. 695–709, 2022.

[9] Y.-H. Chen, S. Spencer, A. Laurence, J. E. D. Thaventhiran, and H. H. Uhlig, "Inborn errors of IL-6 family cytokine responses," *Curr. Opin. Immunol.*, vol. 72, pp. 135–145, 2021.

[10] M. F. Mercogliano, S. Bruni, F. Mauro, P. V. Elizalde, and R. Schillaci, "Harnessing tumor necrosis factor alpha to achieve effective cancer immunotherapy," *Cancers (Basel).*, vol. 13, no. 3, p. 564, 2021.

[11] D. Jang *et al.*, "The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α inhibitors in therapeutics," *Int. J. Mol. Sci.*, vol. 22, no. 5, p. 2719, 2021.

[12] Y. Jiang *et al.*, "STAT1 mediates transmembrane TNF-alpha-induced formation of death-inducing signaling complex and apoptotic signaling via TNFR1," *Cell Death Differ.*, vol. 24, no. 4, pp. 660–671, 2017.

[13] E. Sauer, B. Madea, and C. Courts, "An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensically relevant body fluids," *Forensic Sci. Int. Genet.*, vol. 11, pp. 174–181, 2014.

[14] Y. Guan *et al.*, "Evidence that miR-146a attenuates aging-and trauma-induced osteoarthritis by inhibiting Notch1, IL-6, and IL-1 mediated catabolism," *Aging Cell*, vol. 17, no. 3, p. e12752, 2018.

[15] Y. Cheng, M. Yang, and J. Peng, "Correlation the between the regulation of miRNA-1 in

c-Met-induced EMT and cervical cancer progression," *Oncol. Lett.*, vol. 17, no. 3, pp. 3341–3349, 2019.
[16] X. Zhang *et al.*, "MicroRNA-146a targets PRKCE to modulate papillary thyroid tumor development," *Int. J. cancer*, vol. 134, no. 2, pp. 257–267, 2014.
[17] N. Wang, F.-E. Chen, and Z.-W. Long, "Mechanism of microrna-146a/notch2 signaling regulating il-6 in graves ophthalmopathy," *Cell. Physiol. Biochem.*, vol. 41, no. 4, pp. 1285–1297, 2017.
[18] Y. Zhang *et al.*, "Dynamic monitoring of circulating microRNAs as a predictive biomarker for the diagnosis and recurrence of papillary thyroid carcinoma," *Oncol. Lett.*, vol. 13, no. 6, pp. 4252–4266, 2017.
[19] M. Sun *et al.*, "Associations of miR-146a and miR-146b expression and clinical characteristics in papillary thyroid carcinoma," *Cancer Biomarkers*, vol. 15, no. 1, pp. 33–40, 2015.
[20] S. Ghafouri-Fard, Z. Shirvani-Farsani, and M. Taheri, "The role of microRNAs in the pathogenesis of thyroid cancer," *Non-Coding RNA Res.*, vol. 5, no. 3, pp. 88–98, 2020.
[21] H. Motedayyen, S. Ghotloo, M. Saffari, M. Sattari, and R. Amid, "Evaluation of microRNA-146a and its targets in gingival tissues of patients with chronic periodontitis," *J. Periodontol.*, vol. 86, no. 12, pp. 1380–1385, 2015.
[22] J. Zhou *et al.*, "Dysregulation in microRNA expression in peripheral blood mononuclear cells of sepsis patients is associated with immunopathology," *Cytokine*, vol. 71, no. 1, pp. 89–100, 2015.
[23] M. V Nemtsova *et al.*, "Epigenetic changes in the pathogenesis of rheumatoid arthritis," *Front. Genet.*, vol. 10, p. 570, 2019.
[24] N. J. Béres *et al.*, "Role of altered expression of miR-146a, miR-155, and miR-122 in pediatric patients with inflammatory bowel disease," *Inflamm. Bowel Dis.*, vol. 22, no. 2, pp. 327–335, 2016.
[25] K. Çarman *et al.*, "Expression of MicroRNA 146a, 155, 181 and 223 in febrile seizure," *Turk. J. Pediatr.*, vol. 63, no. 4, 2021.
[26] P. Huang, X. He, and M. Xu, "The role of miRNA-146a and proinflammatory cytokines in carotid atherosclerosis," *Biomed Res. Int.*, vol. 2020, 2020.