Association of MicroRNA-146a expression level and it's Gene Polymorphism rs2910164 C/G in patients with Rheumatoid Arthritis

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Abstract. MicroRNAs are small non-coding RNAs that can regulate a variety of immune functions. MicroRNA146a (miRNA-146a) is regarded as a crucial regulator of posttranscriptional gene expression, suggesting a role in autoimmune disorders. This study was performed to investigate association miRNAs-146a expression and its polymorphisms in patient with Rheumatoid arthritis. The current study includes 120 subjects classified into two main groups: 60 RA patients and 60 healthy controls. Quantitative expression of serum miRNA-146a, as well as its genotyping rs2910164 (C/G) were done to all subjects using real-time PCR and Tetra primer ARMS-PCR respectively. Serum miRNA-146a significantly over expressed in RA patients (fold change 2.59±1.18), compared to the controls (fold change 1.07±0.41), (P=< 0.0001).The receiver operating characteristics (ROC) curve was plotted to compare the expression efficiency of miR-146a showing high sensitivity 91.7 % and high specificity 91.1 %, AUC 0.95, (95% CI 0.91-0.99), the cut off value was (1.57). On the other hand MiRNA-146a (rs2910164) genotyping revealed that no significant variation between the RA patients in the comparison with the control group under all co-dominant model heterozygous genotype (C/G), co-dominant model homozygous genotype (G/G), dominant pattern (C/G+G/G) genotypes, recessive model (G/G) genotype. Analysis of the allele frequencies no statistically significant differences between patients and controls. Conclusion miRNAs-146a expression can be used as diagnostic markers for RA patients and miRNA-146a rs2910164 C/G not associated with RA susceptibility. The C allele of miRNA-146a (rs2910164) can be considered to be protective.

1 Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that primarily affects small joints. It is characterized by inflammation and cellular proliferation in the synovial lining of the joints, which can lead to cartilage and bone destruction.[1]. RA is a chronic

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autoimmune inflammatory disease affected by genetic, epigenetic, and environmental factors. The discovery of novel gene polymorphisms and their association with disease susceptibility have enriched our understanding of the pathogenesis of rheumatoid arthritis (RA) [2]. Locally and systemically, the cellular elements and soluble components of the innate and adaptive immune systems exert direct and/or indirect effects on tissue inflammation and remodeling [2]. Epigenetic changes can influence gene activity and expression without altering the DNA sequence[3]. By regulating the post-translational expression of crucial genes involved in synovial inflammation and joint destruction, epigenetic modifications such as dysregulated micro-RNA (miRs) production, DNA methylation, and histone acetylation also play significant roles in the development of RA and its severity [4, 5]. MicroRNAs are short non-coding RNAs that range in length from 19 to 23 nucleotides. They can regulate mRNA expression by binding to target mRNAs' 3'-untranslated regions (3'-UTRs). MiRNAs' post-transcriptional regulatory roles include mRNA degradation, mRNA decay, and mRNA translation inhibition [6, 7].

MicroRNAs (miRNA) can regulate the expression of numerous messenger RNAs (mRNA), thereby profoundly modulating numerous physiological and pathological responses [8, 9]. Moreover, miRNAs have been identified as potential regulators of genes implicated in the pathogenesis of autoimmune and inflammatory diseases [10]. MiR-146a, a gene associated with the transcription factor NF-B [11], has been extensively studied for its function in innate immunity [12]. This microRNA is essential for the negative regulation of proinflammatory cytokine production, thereby modulating the severity of the inflammatory response [13]. MiRNA-146a may regulate the expression of the TNF- signaling pathway regulators interleukin (IL)-1 receptor-associated kinase (IRAK1), IRAK2, and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [14, 15]. Single nucleotide polymorphisms (SNPs) account for the majority of human gene variations. SNPs in the miRNA loci can cause aberrant miRNA regulation by modifying their expression or maturation [16]. Recent research suggests that miRNA gene polymorphisms, including miRNA-146 rs2910164 and miRNA-499 rs3746444, may be associated with inflammatory arthritis. Numerous studies have investigated the relationship between SNPs in miR-146a and cancer and autoimmune disease susceptibility [17].

2 Materials and Methods

2.1 Patient and control

- **Patient group.** A total of 60 cases of females Rheumatoid arthritis examined during the period between January/ 2022 to April/ 2022. The age of RA individuals between 24 and 75 year. Arthritis patients were attending in Merjan Teaching Hospital, Rheumatology Unit Babylon province and Al-sader Teaching Hospital, Medical Rehabilitation and Joint Unit, Al-Najaf Al-Ashraf province and examined by specialist physicians and diagnosed as rheumatoid arthritis patients depending on the clinical and serological parameters according to 2010 ACR/ EULAR criteria.
- **Control group** composed of 60 female apparently healthy person. The age of the control persons between 24 and 70 year. The samples were taken from person who did not exhibit RA symptoms and have no history of autoimmune diseases during the clinical and serological examination
- **Blood Samples**

Blood from patients and control groups was collected and transferred into two tubes, (1 ml) transferred to EDTA tube in order to extraction of DNA then amplification by PCR for study miR-146a C/G SNP and (3 ml) of blood transferred to gel tube and centrifuged at 4000

rpm for 5 minute to separate serum then the serum was frozen at -20 ºC for determine of microRNA expression level .

2.2 Ethical Approval

The Institutional Ethics Committees of Kufa University's College of Science and AL-Kufa General Hospital have accepted a study concept for human studies. In addition, prior to participating in the trial, each subject provided written, informed consent.

Table 1. Sequence of primers used in present study

Primer	Direction	Sequence	Product
miR-146a rs2910164 C/G SNP	Common Forward	5'TAGACCTGGTACTAGGAAGCAGCTGCAT-3'	445pb
	Common Reverse	5'-GAGTAGCAGCAGCAGCAAGAGAGACTT-3'	
	Primer-F (Allele-C)	5'TCCATGGGTTGTGTCAGTGTCAGAGCTC-3	290pb
	Primer-R (Allele-G)	5'ATATCCCAGCTGAAGAACTGAATTACAC-3'	203pb
miR-146a for q-PCR	Sense	5'TGAGAACTGAATTCCATGGGT-3'	
	Antisense	5'GCAGGGTCCGA GGTATTC-3'	

2.3 DNA extraction and amplification

2.3.1 DNA extraction and purification

DNA extraction FavorPrepTM Blood/ Cultured Cells Genomic DNA Extraction Mini Kit (FAVORGEN-TIWAN) is used for DNA extraction from whole blood. The purity of human DNA was estimated by UV/Visible spectrophotometer at260/280 nm and according to the instruction of extraction kit the accepted absorbance ratio for pure DNA range between 1.7 and 2.1 to give DNA yields about 4-10 µg/ml.

2.3.2 DNA amplification

GoTaq® Green Master Mix (Promega-USA) components, volumes and their concentrations for amplification of MiR-146a C and/or G allele(s) for SNP detection by ARMS-PCR were reported in table (2). Amplification conditions of MiR- 146a C/G SNP were illustrated in table (3).The PCR products were analyzed by agarose gel electrophoresis at75 volts, for 80 minutes. Agarose gel was positioned on the UV trans-illuminator of gel documentation under UV beam and the pictures were taken by means of camera.

Table 2. Mixture of ARMS-PCR for MiR-146 C/G SNP detection

Mixture Solution	Volume	Concentration
Master mix	12.5 µL	1X
Target DNA	5 µL	-
Forward Outer Primer	1.5 µL	10 pmol/ µl
Reverse Outer Primer	1.5 µL	10 pmol/ µl
Forward Inner Primer	1.5 µL	10 pmol/ µl
Reverse Inner Primer	1.5 µL	10 pmol/ µl
Nuclease free water	1.5 µL	
Total volume	25 µL	-

Table 3. Amplification conditions of MiR- 146a C/G SNP

Steps	Temperature	Time	No. of cycle
Initial denaturation	95 ºC	5 minute	1
Denaturation	95 ºC	30 second	35
Annealing	58 ºC	30 second	
Elongation	72 ºC	30 second	
Final elongation	72 ºC	10 minute	1
Hold	4 ºC	30minute	1

2.4 Total RNA extraction of human serum using TRIzol up kit

Serum samples which are frozen at -80 C left to dissolve at room temperature, after complete melting, RNA extraction workflow as follow:

1) A volume of 1ml of TRIzol reagent was added to serum samples in proportion 1:1.
2) Pipetting up and down was done by micropipette until no visible precipitate appeared in lysate.
3) Incubation was for 5 minutes at room temperature.
4) A volume of 100µl of chloroform was added and shaked well by hand for 30 seconds then incubation for 3 minutes at room temperature.
5) Centrifugation at 10000xg was for 15 minutes at 2-8 C. After centrifugation, the mixture was separated into three layers: lower organic pink layer which contain proteins, pale white middle layer contains DNA and colorless upper layer contain total RNA.
6) The upper colorless layer was transferred to a new fresh tube and 250 µl of isopropanol were added and mixed well by hand then incubated at room temperature for 10 minutes.
7) Centrifugation at 10000xg was for 10 minutes at 2-8 C. Then the supernatant was discarded and the RNA can be seen as colloidal precipitate at the walls and bottom of tube.
8) A volume of 1 ml of 75% ethanol was added to the vertexing tube.
9) Centrifugation at 7500xg was for 5 minutes at 2-8 C.
10) The supernatant was discarded and air-dried RNA pellet was for 5 minutes.
11) RNA pellet was dissolved in 50 µl of RNA dissolving solution, and incubated at 55-60º for 10 minutes.

2.5 Estimation of total RNA concentration and purity

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

2.6 Reference Gene Selection

The reference gene or housekeeping gene or endogenous control gene were selected by finding the best and the more stable reference gene expressed in the serum samples. The best reference gene depends on three parameters: high expression level; stable and expressed among all samples; and then showing of converge expression level among all samples [18]

2.7 Determination of miR-146a and U6 reference Expression level 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, 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 were all thawed on ice and each solution was mixed well.
- GoTaq® 1-Step RT-qPCR reaction was prepared, as shown in table (4).
- RT-qPCR reactions were performed using the cycling program shown in table (5).

Table 4. 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)	0.6 µl	300 nM
Reverse Primer (20X)	0.6 µl	300 nM
MgCl2	1.6 µl	25 mM
RNA template	5 µl	100 ng
Nuclease-Free Water	1.8 µl	-

Table 5. One-step RT-qPCR programs.

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

2.8 Calculating Gene Expression (Gene Fold)

Gene expression or gene fold or RQ (Relative quantification) value were calculated by Pfaffl equation [19]:

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

The gene fold was calculated firstly by collecting CT (CT - cycle threshold) average value from real time PC device for each triplicated sample then ΔCT value was calculated for each sample as follows:

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

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 is not affected by experiment.

Calculating ΔΔCT value is found as follows:

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

After calculating ΔΔ CT for all samples, the final equation is taken to calculate the gene expression (fold change) as follows:

$$\mathbf{Fold\ gene\ expression\ RQ = 2^{-(\Delta\Delta CT)}}$$

2.9 Statistical Analysis

The statistics that use in the current study are (mean ±SD, the t-test(independent) and ANOVA test by IBM SPSS version 23. The genotypes data were also analyzed. The level of significance that used was ≤ 0.05 in all statistical analysis. Hardy–Weinberg equilibrium (HWE) HWE can be illustrated arithmetically: p2+2pq+q2 = 1.

Where 'p' and 'q' represent the frequencies of alleles.The multi nominal logistic regression was estimated by SPSS program to evaluate the relationship between genotype and the frequencies of allele in rs2910164 C/G MiRNA146a. The output data analyzed as (OR) , (CI 95%) and P value. This type of statistical analysis and these models were used according to [20]

3 Results

3.1 Detection of rs2910164 C/G MiRNA146a SNP

Tetra-primers for the amplified products of rs2910164 MiRNA146a gene used in T-ARMS-PCR [21]. Common forward (CF) and common reverse (CR) primer set give the amplified product of 445 bp as an internal control. Allele-C Forward and Common Reverse primer set make the product size of 290 bp for allele-C and allele-G Reverse and Common Forward primer pair gives a 203 bp product for allele G. Heterozygous CG genotype showed when primer pair of allele-C (forward) and common reverse primers amplify the allele C and; primer pair allele-G (reverse) and common forward amplify the allele G with internal control (F, R) resulting three bands (445bp,290bp,203bp). In homozygous GG genotype pattern allele-G (reverse) and common forward amplify the product of allele-G and common fragment resulted two bands(445bp,203bp). In homozygous genotype CC genotype primers set of allele-C (forward) and common reverse amplify the allele C and control DNA fragments showing the two bands (445bp, 290bp) as shown in figure (1).

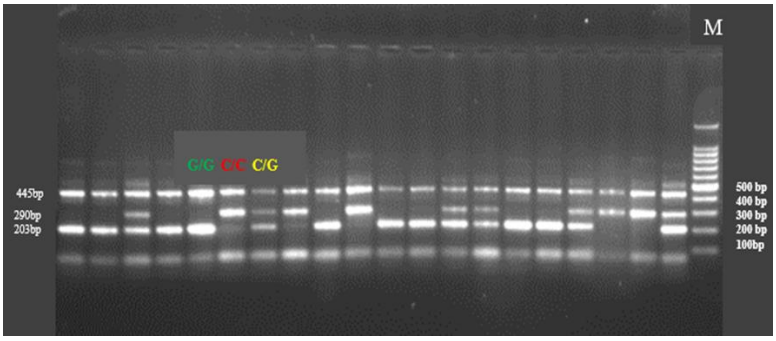

Fig. 1. Agarose gel electrophoresis image of ARMS- PCR products of MiRNA rs2910164 of 146a SNP. M: marker (100bp). Electrophoresis conditions: 1.5% agarose, 80 Min., 80 volt.

3.2 Estimation of genotype and allele frequencies of rs2910164 C/G MiRNA146a gene polymorphisms

Genotype and allele frequency of rs2910164 C/G MiRNA146a gene polymorphisms results were examined by multinomial logistic regression, under codominant, dominant, recessive, and additive models in RA patients and the healthy subjects .The results revealed not significant variation between the RA patients in the comparison with the control group under

all co-dominant model heterozygous genotype (C/G), co-dominant model homozygous genotype (G/G), dominant pattern(C/G+G/G) genotypes, recessive model (G/G) genotype as in table (6). Analysis of the allele frequencies no statistically significant differences between patients and controls as shown in table (7).

Table 6. Genotype of rs2910164 C/G polymorphism for 146a gene of total healthy subjects and total patients group.

rs2910164 C/G 146a	**Control N=60**	**Patients N=60**	**OR (CI 95%)**	**Adjusted RO P value**
Codominant				
C/C	8	10	1.0	
C/G	26	26	0.8 (0.27 to 2.35)	0.68
G/G	26	24	0.74 (0.25 to 2.18)	0.58
Dominant				
CG+GG	52	50	0.77 (0.28 to 2.11)	0.61
C/C	8	7	1.0	
Recessive				
C/C+C/G	34	36	1.0	
G/G	26	24	0.87 (0.42 to 1.80)	0.7
Additive				
C	42	46	1.0	
G	78	74	0.86 (0.51 to 1.47)	0.6

Table 7. MiRNA146a allele frequency

MiRNA146a allele frequencies (n=120)						
	All subjects		**Group=Control**		**Group=patient**	
Allele	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
G	152	0.63	78	0.65	74	0.62
C	88	0.37	42	0.35	46	0.38

3.3 Concentration of miRNA-146a in serum of studied group

MiRNA-146a gene expression was considerably increased in the RA patients (fold change 2.59±1.18), compared to the controls (fold change 1.07±0.41), (P=< 0.0001), as in figures (2) and (3).

Sensitivity and specificity of miRNA-146a were evaluated by receiver operating characteristics (ROC) curve of MiRNA146a. The cutoff value was 1.57, sensitivity 91.1 %, specificity 83.0 %, AUC 0.95, and (95% CI 0.91-0.99) shown in figure (4).

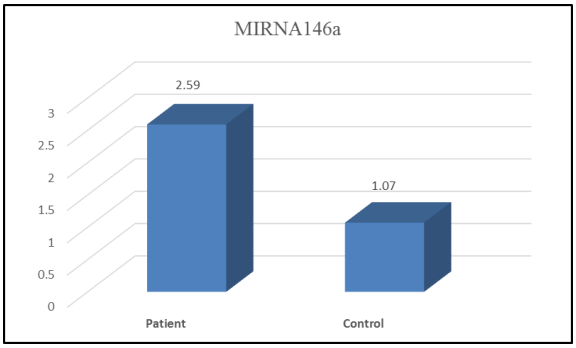

Fig. 2. The fold change difference of miRNA-146a between RA patients and control.

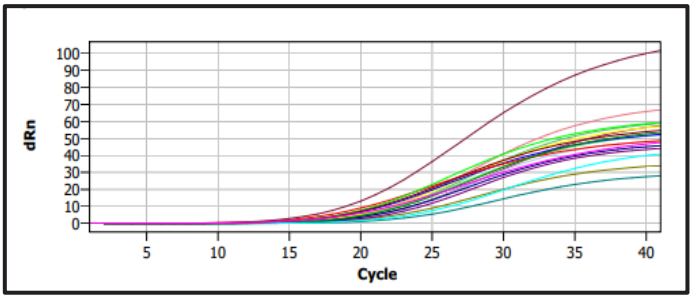

Fig. 3. Real time PCR image show Ct value of miRNA-146aand U6 genes.

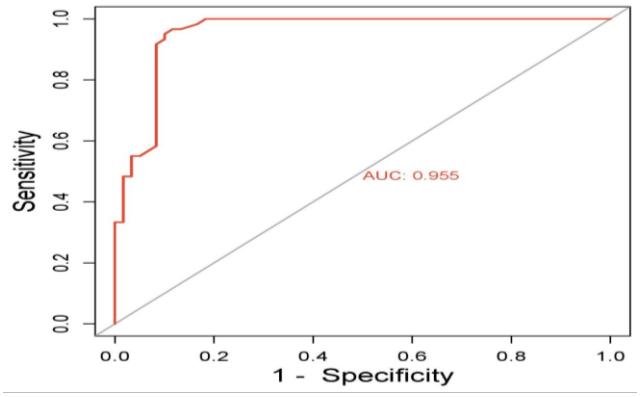

Fig. 4. Receiver operating characteristic (ROC) curve of miRNA-146a.

4 Discussion

4.1 Association of MiRNA146a rs2910164 C/G gene polymorphisms with rheumatoid arthritis

Because miRNAs appear to be relevant in RA genetic association studies, polymorphisms in these miRNAs and their target genes are also important and should be explored for their role in RA risk. In the present the results revealed not significant variation between the RA patients in the comparison with the control group under all co-dominant model heterozygous genotype (C/G), co-dominant model homozygous genotype (G/G), dominant pattern

(C/G+G/G) genotypes, recessive model (G/G) genotype. These result compatible with results obtain by Amal et al., [22] who did not find any significant association of miRNA-146a polymorphism (rs2910164) with RA risk, severity, and activity in Egyptian female patient. Indeed, our findings demonstrated that the G allele was more common in patients than the C allele, although there were no significant differences between them. This finding is consistent with other studies that found the G/C genotype and G allele to be the most common in patients, but there was no significant difference in the frequency distribution of genotypes and alleles between patients and controls (13) and Ciccacci, et al., [23] that the C allele of rs2910164 in miR-146a has a protective function in the etiology of RA.The similar finding was found in an Iranian investigation that found no link between the MiRNA 146a rs2910164 variation and RA susceptibility [24]. Shaker et al [25] found the same thing in the Egyptian population, as did Hassine, et al., [26], who discovered that the G allele of miRNA-146a (rs2910164) was considerably research, such as (Ayeldeen1 et al., [27] found that miRNA-146a (rs2910164) genotyping in RA patients revealed that the G/G genotype was considerably greater in RA patients compared higher in RA patients than in controls. Zhou, et al., [28] discovered an association between the miR-146a rs2910164 genotype G/G and an elevated risk of RA in Chinese female patients. Individuals bearing the G/C versus G/G genotype in the Iranian population, as well as the G/C + C/C versus G/G genotype in females alone rather than males, are at risk of getting RA [29]. The rs2910164 polymorphism had a direct functional effect on miR-146a's ability to inhibit its target genes TRAF6 and IRAK1[30]. Given that TRAF6 and IRAK1 have been linked to RA pathogenesis, this polymorphism may play a role in RA development [28].. In contrast, Zhou et al. reported that the C/C genotype in miRNA-146a (rs2910164) may affect disease activity when measured with the DAS28 score. Additionally, (Slaker et al., 2018) the C/G and G/G genotypes of miRNA-146a were associated with positive erosions (25). MiRNA-146a may be a potential biomarker for extra-articular manifestations of RA [31]

4.2 Association of MiRNA146a gene expression with rheumatoid arthritis

MiRNA-146 (microRNA-146) is an important regulatory molecule involved in the immune response and inflammation. It plays a role in various autoimmune diseases, including rheumatoid arthritis (RA). The irregular expression of different miRNAs, as epigenetic regulators of biological signaling pathways, has a strong relationship with some inflammatory diseases and autoimmunity [32, 33]. Recent studies have revealed the possible effects of altered miRNA expression profile in the pathogenesis of RA.

In fact, dysregulation of miRNA expression provides important information about the development of RA [34]. In the current study MiRNA-146a gene expression was considerably increased in the RA patients (fold change 2.59±1.18), compared to the controls (fold change 1.07±0.41), (P=< 0.0001). This results agreement with other results in Egyptian population, patient fold change (0.958, 25.14±18.23, 11.52 ± 11.43), Control fold change (0.242, 2.07±0.62 and 1.01 ± 0.48), (P vlue = 0.036), (P=< 0.0001) and ($p<0.001$) respectively obtained by El-bakry et al., [35], Elmalt et al., [36] and Elsayed et al ., [37] they are reported highly significant statistical differences between patients and healthy controls as regards miR-146a relative expression. Other studies reveled similar results like Rezaeepoor, et al., [38] who finding all miRNAs (miR-155, miR-150, miR146a, miR-146b, miR-125a-5p, and miR-223)that used in this study increased in both groups of RA patients were significantly overexpressed in both groups(poor and good response of RA patients to therapy compared to healthy control. Other study obtained by (Pauley, et al., [39] demonstrate that RA patient PBMCs exhibit elevated miRNA expression in a similar manner to RA synovial tissue, with a 2.6-fold change. The upregulation of miRNA-146 in RA suggests its involvement in the disease process.

Conversely, other studies have reported downregulation of miR-146a in RA patients. These studies have suggested that reduced expression of miR-146a may contribute to the sustained activation of pro-inflammatory pathways in RA, leading to increased cytokine production and joint inflammation [40]Wang et al., 2012 [41] detected that circulating levels of miRNA-146a-5p were significantly down regulated in PBMCS in RA patients.The exact reasons for the discrepancies in miR-146a expression levels among different studies are not fully understood.

It could be due to differences in patient populations, disease duration, sample types, or technical variations in the experimental procedures. The receiver operating characteristics (ROC) curve was plotted to compare the expression efficiency of miR-146a the current results showing high sensitivity 91.1 % and specificity 83.0 %, AUC 0.95, (95% CI 0.91-0.99), the cutoff value was 1.57. This result consistent with previous studies like Elsayed et al., [37] who showing the highest sensitivity and specificity (96% and 100%, respectively) (AUC: 0.992 at a cut off value of 2.16) where's Elmalt et al., [36] showing both sensitivity and specificity were 100%. This suggests that miR-146a was a good diagnostic RA biomarker, which is consistent with previous research that found higher miR-146a expression in RA patients' synovial fluid and whole blood.

5 Conclusion

This study concluded that miR-146a expression was considerably higher in the serum of RA patients compared to healthy controls, implying that miR-146a expression can be employed as a diagnostic marker for RA patients. MiRNA-146a rs2910164 C/G not associated with RA susceptibility. The C allele of miRNA-146a (rs2910164) can be considered to be protective.

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