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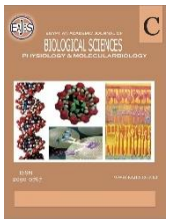

Evaluation of miRNA- 223 Expression in Patients with Systemic Lupus Erythematosus

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by dysregulated innate and adaptive immune responses. Eighty cases of SLE involving 40 patients with lupus nephritis with a mean age (33.80±11.824) years (13 males and 27 females) and 40 patients with lupus arthritis with a mean age (34.43±11.942) year (10 males and 30 females). Also,40 (33±12.077) healthy control. There was found a significant decrease (P< 0.001) in miRNA-223 levels in patients with lupus nephritis the mean fold change (0.11±0.22) compared to the controls (1.02±0.188). miRNA-223 was significantly down-regulated (P < 0.001) in patients with lupus arthritis (0.16±0.229) compared to the controls (1.02±0.188). According to gender, there is a difference in microRNA- 223 levels between males and females, and there is a significant association with gender (P<0.001) in lupus nephritis, mean fold change in males and females (0.34 ± 0.28) and (0.007±0.003) respectively, while in lupus arthritis mean fold change (0.36±0.15) and (0.1±0.04) in males and females respectively with (P<0.001). The cases are divided into four groups according to age, into four age groups, miR-223 levels significantly correlated with age, (P=0.028) in lupus nephritis and (P=0.01) in lupus arthritis. The sensitivity and specificity of miRNA-223 were 100% in lupus nephritis, The Receiver operating characteristic (ROC) curve showed (AUC) was (1.000) and a cut-off point of (0.6716). Also, the sensitivity and specificity of microRNA-223 in lupus arthritis were 100%. ROC observed (AUC) was (1.000) and cut off (0.6933).

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by autoantibody production and multisystem inflammation (You *et al.*, 2021). It affects more than one organ such as serous membranes, the kidney, skin, joints, the central nervous system, and hematological, gastrointestinal, and cardiovascular systems (Barturen *et al.*, 2021 Karnal *et al*., 2020) The incidence and prevalence vary widely with sex, age, and ethnicity. It is more prevalent in women (about nine times more than men) and more diagnosed between 15 and 44 years old (Sarray *et al.*, 2020). The incidence rate of SLE worldwide is about 20–70 per 100,000 general population **(**Barber *et al*., 2021). These autoantibodies mainly act against intracellular components in the nucleus, such as antinuclear antibodies (ANA), double-stranded (dsDNA) DNA, and single- (ssDNA). (Yaniv *et al.*,2015, Fattal *et al.*,2010).

MicroRNAs are a family of short single-stranded non-coding RNA molecules that regulate post-transcriptional gene silencing through the base-pair binding on their target mRNAs (Bartel,2004), thereby regulating most if not all cellular and biological processes (Gurtan *et al.*,2013). It is known that miRNAs can influence the growth, differentiation, and death of cells and are thus involved in the pathogenesis of many diseases (Beermann *et al.*, 2016). Many immunoregulatory genes are miRNA targets, including transcription factors, cofactors, and chromatin modifiers, and some have binding sites for eight or more different miRNAs. Similarly, each miRNA has the potential to recognize dozens, if not hundreds, of target genes. As a result, miRNA dysregulation of their expression can affect the occurrence and development of autoimmune diseases through different pathways including the release of inflammatory mediators, innate immune responses, lymphocyte function, the signaling of toll-like receptors (TLRs), and nuclear factor (NF)-kB (Long *et al.*,2018, Yan *et al.*, 2014).

MATERIALS AND METHODS

Patient and Control:

The blood samples were collected from 40 patients with lupus nephritis (13 male and 27 female), and 40 patients with lupus arthritis (10 male and 30 female) who came to Al-Sader Medical City, Najaf province. A case-control group (40) samples (13 male and 29 female) without a history of autoimmune diseases has been conducted. Patient groups were diagnosed in accordance with the classification criteria of the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR).

Total RNA Extraction:

Using a Triazole Reagent kit, Total RNA was isolated and purified from the whole blood of 120 samples which was done according to the manufacturer's protocol (Invitrogen, USA), and then total RNA concentration and purity were Estimation by using a UV/Visible spectrophotometer instrument. RNA should be stored at –80º.

miRNA-223 and U6 reference gene quantification using RT-qPCR following RNA purification, miRNA profiling can be carried out by Primer sequences used for RT-qPCR analysis as in Table (1). Reaction volumes were set according to recommendations by the manufacturers (GoTaq® 1-Step RT-qPCR kit, Promega) as in Table (2), and the Reaction conditions were set as the following in Table (3).

Table 1: The sequence of primers used in the recent study.

Primers of miRNA	Sequence 5' to 3'
microRNA-223 Forward primer	GCGGCGGTGTCAGTTTGTC
microRNA-223 Reverse primer	GTGCAGGGTCCGAGGT
U6 Forward primer	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT
U6 Reverse primer	CTCAACCTACAATCAAAAACAACACAAACA

Table 2: 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	1.5 µl	300 nM
Reverse Primer	1.5µl	300 nM
RNA template	4 µl	100 ng
$Mgcl_2$	1.6	≥2mM
Nuclease free water	1	
Final volume	20	

Table 3: One-step RT-qPCR programs.

Step	Temperature	Duration	Cycles
Reverse transcription	37℃	15 min	1
Reverse transcriptase inactivation /and start activation of GoTaq DNA Polymerase.	95℃	10 min	1
Denaturation	95℃	10 sec	45
Annealing and data collection	58℃	30 sec	
Extension	72℃	30 sec	
Melt Curve	60-95°C	15 sec	1

Calculating Gene Expression (Gene Fold):

The expression of miRNA-233 for patients' blood samples was normalized to (RNU6-2) reference genes and compared with those in relatively normal healthy control. Finally, calculate the fold-change between the healthy control and patient by using the $2^{-\Delta\Delta ct}$ method described by Schmittgen and Livak (Schmittgen & Livak,2008).

Statistical Analysis:

Experimental results are expressed as mean SD. Statistical analyses for quantitative RT-PCR were performed with the One-way ANOVA and, the chi-square test was used to compare all pairs of groups in a recent study. It was decided to use receiver operating characteristic (ROC) analysis to calculate a parameter's sensitivity and specificity.

The SPSS statistical package for the Social Sciences was used to analyze the results (version 20.0 for Windows, SPSS, Chicago, IL, (USA), (Iuliano & Franzese, 2011).

RESULTS AND DISCUSSION

The age range in the recent study involving (10-50) years for patients with lupus nephritis with a mean age of (33.80 ± 11.824) years and the age range for patients with lupus arthritis (10-50) years with a mean age of (34.43± 11.942) years, the healthy control group's mean age was (33±12.077) years. As seen in Figure (1), the study revealed that there is no significant difference (P=0.97) between the lupus nephritis and lupus arthritis patients and the control group.

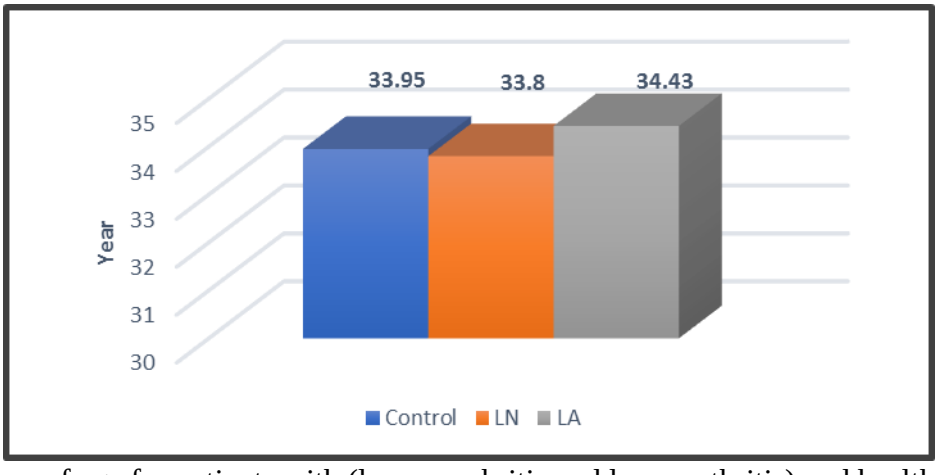

Fig. 1: Mean of age for patients with (lupus nephritis and lupus arthritis) and healthy controls.

Identification of the Expression Levels of miR-223 in SLE Patients And Control:

According to the results of the current investigation, miR-223 expression levels were significantly diminished ($p < 0.001$) in lupus nephritis patients (mean fold change = 0.11±0.22) as compared to controls (mean fold change = 1.02±0.188). Additionally, the study found that patients with arthritis had significantly lower levels of microRNA-223

in lupus nephritis patients (mean fold change = 0.16±0.229) than controls (mean fold change = 1.02±0.188) ($P < 0.001$) as in Figure (2). Down-regulation of miR-223 was reported among SLE patients compared to controls and lower values were reported among the LN group compared to the non-LN group (Abdul-Maksoud *et al.*,2021). In SLE patients, the expression of miRNA-223 in peripheral plasma was significantly decreased in SLE patients with active nephritis (Carlsen *et al.*,2013).This change and decrease in expression levels are caused by many factors. Growing evidence shows that exposure to environmental stimuli can cause a variety of epigenetic modifications, including miRNA expression, global or gene-specific DNA methylation modifications, and histone changes (Collotta *et al.*, 2013, Mahna *et al.*,2021). Significant differences in miRNA profiles have been observed between farmworkers with exposure to insecticides (organophosphates) and non-farmworkers, as well as between farmworkers during thinning and postharvest agricultural seasons. And, there is a positive dose-response relationship between certain miRNAs and organophosphate insecticide metabolites in farmworkers (Weldon *et al.*,2016). Different studies showed the effect of drugs on the expression of miRNAs. One study aimed to evaluate the side effects of escitalopram drug on miRNA expression, which identified a relationship between side effects and changes in peripheral expression of miRNAs, there is an overexpression of miR-185-5p during escitalopram treatment of Major Depressive Disorder (Yrondi *et al.*,2020).

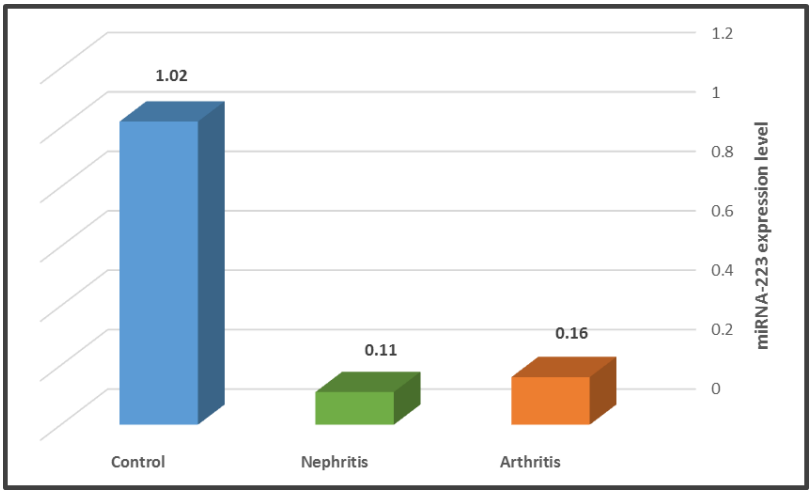

Fig. 2: expression level of miRNA-223 in patients and control.

Identification of the Levels of miRNA-223 According To Gender:

Table 4 shows the difference in expression levels of microRNA- 223 between males and females, RT-PCR analysis indicates there is a significant association with gender ($P<0.001$) in lupus nephritis and lupus arthritis as shown in Table (4), Figure (3), and Figure (4) respectively. The current study indicates that the difference in expression levels between males and females may be related to the X chromosome. The human X chromosome is highly enriched in miRNAs as compared to the Y-chromosome, a more recent study, through the map of microRNA sequences on the human X chromosome. The x chromosome contains an unexpectedly high number of miRNAs (118), in comparison to the Y chromosome (4 miRNA sequences predicted) (Di Palo *et al.*,2020). Moreover, there is evidence that men and women express different miRNAs in different tissues, which is linked to X chromosome genes. Additionally, the sex-biased expression of miRNAs may have functional consequences. (Dai & Ahmed,2014). So the severity and etiology of the disease vary between men and women.

and numerous autoimmune diseases affect women more frequently than men (Voskuhl,2011, Hewagama *et al.*,2013, Koch-Henriksen *et al.*,2010, Amur *et al.*,2012). Among the X-linked miRNAs involved in immune regulation, miR-223 is probably the most studied so far. miR-223, which is involved in autoimmune disorders (Fulci *et al.*,2010). Other X-linked miRNAs include (miR-20a/b, miR-106 a/b, miR-513, and miR-424) they considered an example of a miRNA-dependent, sex-specific regulation of immune responses and cancer immunosurveillance is represented by the PD-1/PD-L1 pathway. directly targets the stability and/or the translation of PD-L1 transcript or interferes with the transcription factors modulating its expression (Carè *et al.*,2018). The involvement of X-linked miRNAs in gender-biased immunity is also supported by a study reporting differential expression of six X-linked miRNAs (miR-221, miR-222, miR-98, miR-532, miR-106 and miR-92a) in PBMC (peripheral blood mononuclear cells) between males and females affected by rheumatoid arthritis, an autoimmune disease that affects females three times more often than men (Khalifa *et al.*,2016). So that some studies indicate that females are more susceptible to developing lupus than males, and one reason for this may be related to the increased expression of miR-98, miR-188, miR-421, and miR-503 in CD4+ T cells of females than males (Hewagama *et al.*,2013). There are many studies that indicate that lupus erythematosus is more common in women. According to one research by Mobini et al., 95.8% of all SLE cases were women (Mobini et al., 2018). Additionally, indicated that over 92% of women frequently get SLE disorders. (Sassi *et al.*,2017). By comparing the expression patterns of miRNA in males and females, we can better understand the variances in the incidence of SLE disease among women.

Table 4: Fold change difference of miRNA-223 between male and female patients.

Gender	Count	% Within SLE	Mean ± SD	P-value
Male lupus nephritis	13	32.5%	0.34 ± 0.28	0.001
Female lupus nephritis	27	67.5%	0.007 ± 0.003	
Male lupus arthritis	10	25.0%	0.36 ± 0.15	0.001
Female lupus arthritis	30	75.0%	0.1 ± 0.04	

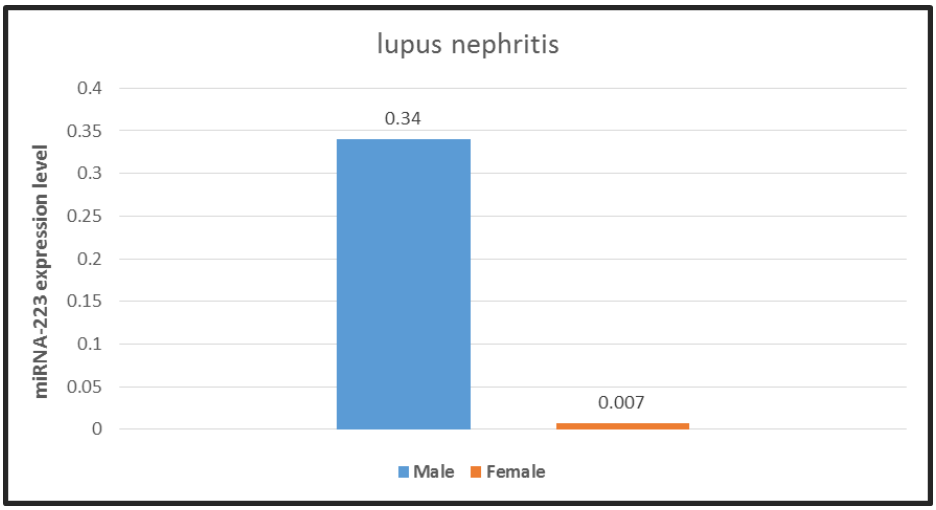

Fig.3: Fold change difference of miRNA-223 between males and females in patients with lupus nephritis.

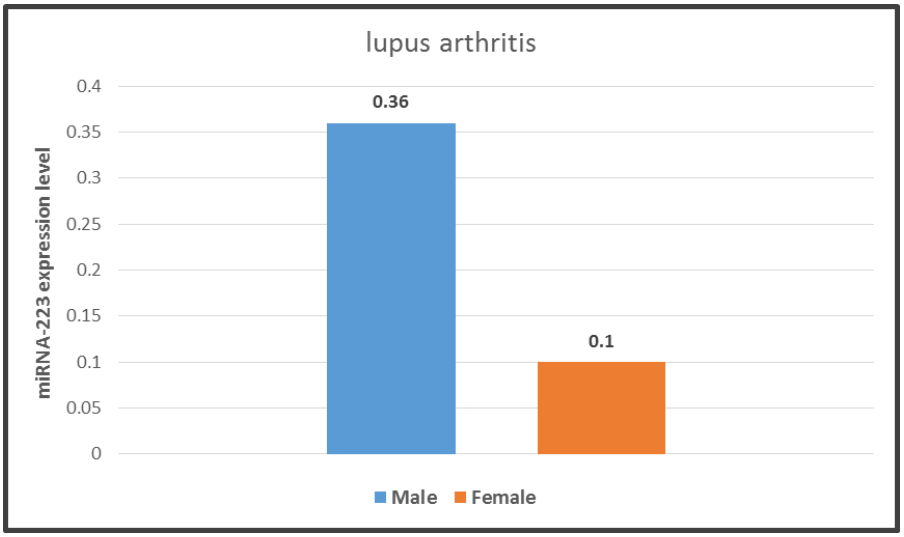

Fig.4: Fold change difference of miRNA-223 between male and female patients with lupus arthritis.

Identification of the Expression Levels of miR-223 According To Age:

All the cases of the recent study are divided into four groups according to age, (10-20) (21-30), (31-40), and (41-50). The results show there is no interaction between age and evaluation of miR-223 expression levels, the data shown to be no significant with aging (P=0.028) in lupus nephritis and (P=0.01) in lupus arthritis as shown in Table (5), and Table (6) respectively. However, our current study discovers a relationship between age and different levels of expression, and this was in agreement with some other studies that showed that miRNA profiles are strongly associated with age, for example, the researchers found that miRNA is altered with age in a human population (Noren *et al.*,2010). A study by Fehlmann *et al.*, observed that age has a greater relationship with 1568 miRNAs profiles (Fehlmann et al.,2020). Research by Ong et al. identified 27 miRNAs of which the expression levels were changed with increasing age in bronchial biopsies (Ong *et al.*,2019). In all asthmatics, the serum miRNA-106a and miRNA-126a expression levels correlated with age (Wardzyńska *et al.*,2021).

Table 5: expression levels of miR-223 depending on age in lupus nephritis

Years	Nephritis (n) Mean ± SD	P-value
10-20	0.19 ± 0.15	0.028
21-30	0.18 ± 0.17	
31-40	0.06 ± 0.13	
41-50	0.05 ± 0.06	

Table 6: expression levels of miR-223 depending on age in lupus arthritis

Years	Arthritis (n) Mean ± SD	P-value
10-20	0.24 ± 0.17	0.01
21-30	0.16 ± 0.05	
31-40	0.11 ± 0.01	
41-50	0.08 ± 0.05	

Receiver Operating Characteristic (ROC) Curve:

High specificity and sensitivity are determined by operating characteristics (ROC) curve analysis. the receiver operating characteristics (ROC) curve analysis for lupus nephritis observed the specificity and sensitivity of miRNA-223 were 100 % and 100%, Area Under the Curve (AUC) 1.000, and the cutoff value was 0. 0.6716 shown in (Table 7) and Figure (5). The receiver operating characteristics (ROC) curve for lupus arthritis showed an Area Under the Curve (AUC) of 1.000, and the cutoff value was 0.6933. The specificity and sensitivity of miRNA-20a were 100% and 100% respectively, shown in (Table 7) and Figure (6). In our study, ROC curve analysis was used to determine differences between patients and control and help us to use miRNA-223 as a diagnosis biomarker.

Table 7: Sensitivity and specificity of miRNA-20a in SLE patients (Lupus nephritis and arthritis).

Parameter	Cut-off	AUC	Sensitivity%	specificity%	P-value
miRNA-223 Lupus nephritis	0.6716	1.000	100 %	100 %	0.001
miRNA-223 Lupus arthritis	0.6933	1.000	100 %	100 %	0.001

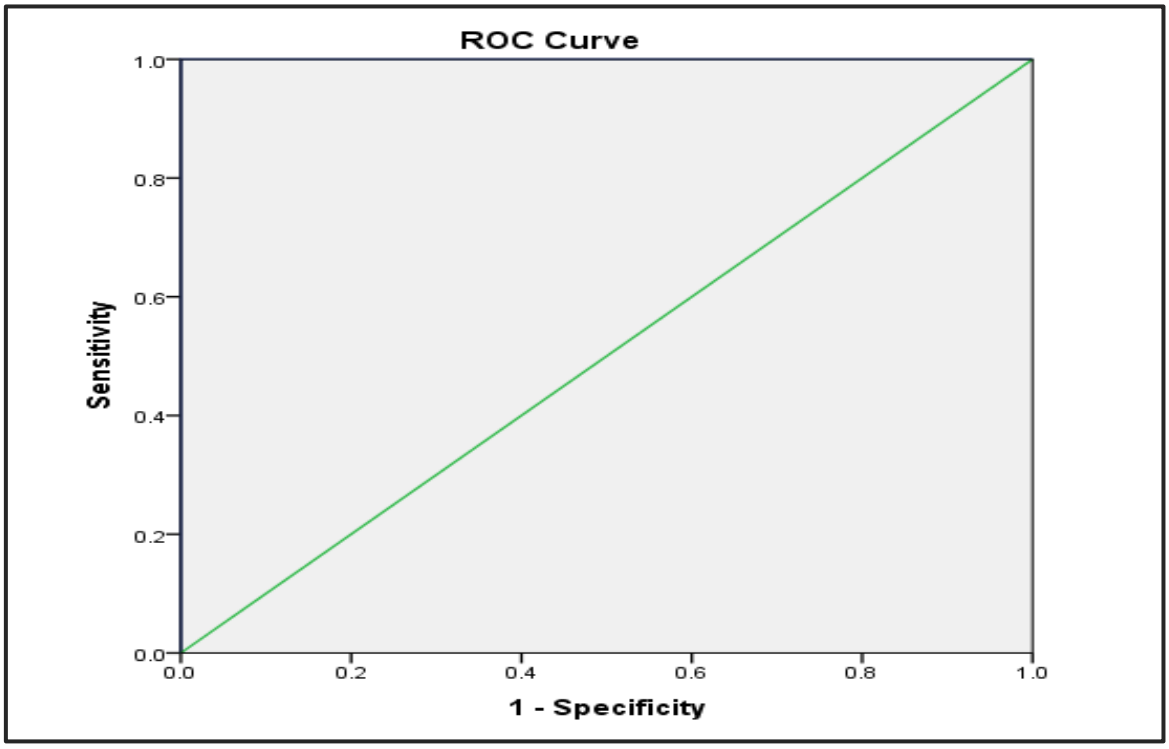

Fig. 5: Receiver operating characteristic (ROC) curve analysis of miRNA-20a in patients with lupus nephritis versus control as a potential biomarker.

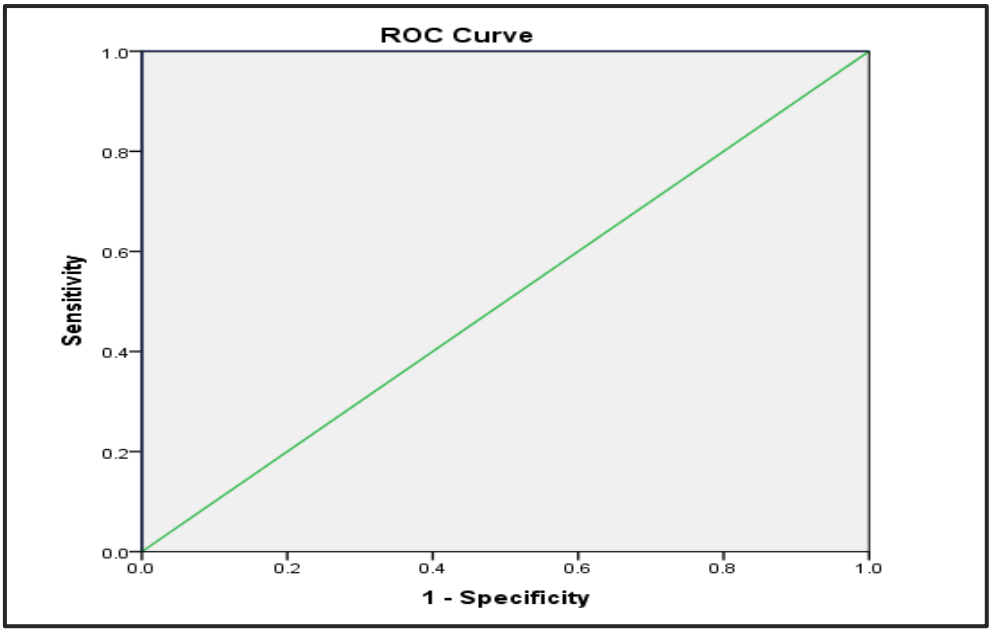

Fig. 6: Receiver operating characteristic (ROC) curve analysis of miRNA-20a in patients with lupus arthritis versus control as a potential biomarker.

Conclusions

In conclusion, miRNA-223 might have a diagnostic value to differentiate patients with SLE from healthy controls so the findings may serve as a potential genetic marker for SLE disease and this study may provide useful information for future research that targets miRNAs as a molecular marker for medical fields, also might serve as a therapeutic target for treatment.

Declarations:

Ethical Approval:Ethical Approval is not applicable.

Competing interests: The authors declare no conflict of interest.

Authors Contributions:I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

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