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Determination of miRNA -125b expression in Ankylosing Spondylitis

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ABSTRACT

Background: The most prevalent kind of spondylo-arthropathies is ankylosing spondylitis (AS); >90% of those who have it are HLA-B27 positive. AS may be influenced by non-HLA genes. MicroRNAs are 18-23 nucleotide non-coding RNA molecules that gene expression control post-transcriptional.

Objective: The aim of the present study is to evaluate the expression profile of miRNA-125b and serum levels of ESR, CRP in the control group and patients alike and find their association with disease progression in AS patients and evaluate their significance as novel markers for AS.

Patients and methods: This case control study included 100 AS male patients and 100 healthy controls of matched age with no history of infectious or autoimmune disorders. AS patients were recruited from the Hematology Clinic in Medical City (Baghdad Hospital) and diagnosed with AS on the basis of complete blood picture (CBC), CRP, along with MRI, and X-Ray. Clinical assessment, history of treatment administration, disease duration, and smoking were taken into account for Disease Activity Indexes for AS patients. Disease was assessed using the Bath AS Disease Activity Indicator (BASDAI), along with ESR (mm/h) and (CRP) (mg/l) to assess the mobility and functional limitations. We investigate (miRNA-125b) expression in individuals with AS and in healthy individuals by real-time quantitative polymerase chain reaction. Characteristic Receiver operating (ROC) in AS patients was done to evaluate accuracy of diagnostic of miRNA-125b in patients with AS from control subjects.

Results: AS male patients had mean age of 40.56 (SD 10.19) and disease duration of 9.04 (SD 2.03) years. The age of the male controls (38.78 ± 10.57 years) was not significantly different from that of AS patients (p=0.227). Both AS Disease Activity Index (BASDAI) and AS Functional Index (BASFI) values were 3.45 (SD 2.03) and 3.84 (SD 2.62), respectively. It was observed that 57% of patients have HLA-B27positive and ESR and levels C-reactive protein were 18.34 (SD 3.45) and 25.39 (SD 19.59) mm/h, respectively. Our results revealed a significant decrease in (miRNA-125b) expression with fold change (0.133) in AS patients. The area under curve (AUC) was 0.962 and cut off point was 8.94. Specificity and sensitivity of miRNA-125b were 92% and 91%, respectively.

Conclusion: Expression profile of miRNA-125b can be used as novel markers for AS.

Keywords: Non-coding sRNA, Disease duration, miRNA-125b, Disease activity, University of Baghdad.

INTRODUCTION

Ankylosing spondylitis is an inflammatory condition (AS), initially the spine joints and a characteristic backache. Permanent deterioration to the axial joints' structure and functionality gradually occurs as the disease progresses, causing a severe morbidity [(1)].

Despite the fact that there is no scientific test that can identify AS, 90–95% of AS patients in Central Europe and North America carry the HLA-B27 gene [(2)]. However, it is still largely unknown what other genetic changes associated with AS exist outside of HLA-B27. The main criteria for diagnosing AS often include HLA-B27, markers of inflammatory, subjective symptoms, and imaging testing. To decrease the time, it takes to diagnose AS, diagnostic indicators with high specificity and sensitivity are urgently needed [(3)].

ESR and CRP values are only high in 50–70% of patients with active illness [(4)]. Studies have shown that histone changes, DNA methylation, and microRNA are some of the epigenetic pathways that support AS (miRNA) [(5)]. Endogenous non-coding RNAs having a length of 18 to 22 nt are known as microRNAs [(6)].

Two-thirds of the human protein-coding genes seem to be under the control of miRNAs. Several findings claim that miRNAs play a crucial part in a variety of physiological and pathological processes [(7)]. Furthermore, miRNAs are highly stable, easy to quantify, and present in a several clinical samples, which makes them excellent diagnosis as invasive-free biomarkers for the primarily diagnosis or prognosis of a number of human diseases, involving Cancer, cardiac disease, rheumatic-related disease, mass cell carcinoma, and cancer of the colon [(8)] .

MiR-125b controls biological processes important for both lymphoma and RA, such as inflammation, B-cell differentiation, TNF production, and apoptosis, as it is an evolutionarily conserved miRNA [(9)].

The major regulators of innate immune and inflammatory reactions have been noted as miRNA-125a and miRNA-125b. Nuclear factor Kappa b (NF-KB) is activated by miRNA-125b in RA, which causes inflammation [(10)].

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The current investigation's goal is being to shed light on this subject by broadening our knowledge of the association between miRNA -125b and AS among male patients from Iraq.

PATIENTS AND METHODS

Patients and controls

Male patients totaling 100 with AS with age ranging from 20 to 45 years old were recruited from the Hematology Clinic in Medical city (Baghdad Hospital) and diagnosed with AS on the basis of complete blood picture (CBC), CRP, along with MRI, and X-Ray.

Clinical assessment, history of treatment administration, disease duration, unique behaviors, particularly smoking, were all taken into account for Disease Activity Indexes for AS patients such as disease was assessed using the Bath AS Disease Activity Indicator (BASDAI), along with ESR (mm/h) and (CRP) (mg/l) was used to assess the mobility and functional limitations [(11)]. Also 100 healthy controls of matched age involved in this study had no history of infectious or autoimmune disorders.

Blood collection

Five milliliters of samples were taken from every patient, every control, and the blood was drawn were placed into tubes contain 1 ml lysis buffer for genetic analysis and stored at -20c for further steps.

miRNA extraction and analysis

Using the EasyPure® Blood Genomic miRNA Kit, whole blood samples from the patient and a healthy control were used to extract miRNA. Using the spectrophotometer Nano Drop (Q5000), miRNA samples were subjected to miRNA quantitation and purity evaluation. The purity of miRNA that is accepted is between 1.7 and 2.

Primer design and its preparation for miRNA-125b

The NCBI Gen Bank database was used to obtain the cDNA sequences of the miRNA-125b gene, as well as miRNA -*U6* as a housekeeping gene.

As directed by the manufacturer, using nuclease-free water, to create solution of a stock with a level of (100 M) for each of the lyophilized primers, they were broken which was then kept at (-20°C).

10 ml of primer stock solution were diluted with 90 ml of nuclease-free water to produce a 10 mM-concentrated working solution, which was then kept at -20 C until use. **Table 1** displays the sequences of primers utilized in this investigation.

Table (1): Primers of miRNA- 125b and used in the study

Primer	Sequence (5′→3′ direction)
miRNA 125b	
miRNA-125b	GGATTCCCTGAGACCCTAAC
RT-miR-125b	GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCCTGGATAC GACTCACAAG
Universe miRNA reverse	GTGCAGGGTCCGAGGT
miRU6 F.P.	A GAGA AGATTAGC ATGGCCCCT
miRNA-Universe, R.P.	GCGA GCAC AGAATTA ATACGAC

Synthesis of cDNA from miRNA-125b

The EasyScript® SuperMix technique for the cDNA synthesis was put through step gDNA elimination and cDNA synthesis to testing. (Transgen, China) was used Reverse transcription reactions should be built in an environment free of RNase, therefore mix tubes were arranged on a PCR tube rack to evaluate the expression of miRNA-125b. As shown in **Table 2**, the reaction component was introduced to the mix tube.

Table 2. The reverse transcription procedure's reaction volume and constituent parts used to create cDNA from miRNA

Component	Volume (µl)
overall miRNA	5
0.5 µlg/l Anchored Oligo (dT)18 Primer	1
Primer Random (0.1 µg/µl)	1
2×EX React Mix	10
Easy Script®RT/RI Mix Enzyme	1
gDNA Remover	1
free Water of RNase-	1
Overall volume	20

As shown in **Table 3**, tubes were put in a thermal cycler program. Synthesized cDNA was either frozen or used right away as a PCR template at -20°C for long-term preservation.

Table 3: Thermal cycler steps of conditions cDNA reverse transcription

Variable	Step 1	Step 2	Step 3
Temp/ °c	25	42	85
Time	10 min	15 min	5 seconds
	Primer Random (N9)	Oligo Anchored (dT)18	Inactivate RTE

Time Quantitative PCR (qRT–PCR):

Levels of miRNA-125b were assessed using reverse transcription-quantitative polymerase chain reaction, a sensitive method for estimating miRNA levels in steady-state samples. To validate the expression of target miRNA, qRT-PCR SYBR Green test (TransStart® Top Green qPCR SuperMix for gene expression) was utilized. In accordance with the manufacturer's instructions, this operation was performed in a volume of 20 µl. (Transgen, China). qRT-PCR with the Cephied, time PCR System (Smart cycler Technologies) and qPCR soft software. Using the 2xqPCR Master Mix Kits components, the expression levels and fold change were measured by measuring the threshold cycle (Ct). Every reaction was performed twice. The appropriate volume of each component was estimated employing **Table 4**.

Table 4: Quantitative real-time PCR components utilized in a gene expression experiment.

Component	Volume (µl)
Master mix Syper Green	10
Forward primer	1
Revers primer	1
CDNA	3
Nuclease free water (N.F.W)	5
Total volume	20

The protocol for cycling was programmed with the following optimum cycles and thermal profile, as shown in **Table 5**.

Table 5: Expression of Genes' thermal Profiles.

Stages	Processes	Temperature	Time	Cycle
Stage 1	Initial Denaturation	94ºC	30 sec	1
Stage 2	Denaturation	94ºC	5 sec	40
	Annealing	56ºC	10 sec	
	Extension	72ºC	20 sec	
Stage 3	Melting curve	65-95 ºC	1sec	1

For each sample, the threshold cycle (CT) was using the real-time cycler program to determine Normalization of gene expression data against housekeeping genes. The finding was reported as folding alters the expression of genes using the ΔΔCt technique by [12], which was employed as advised for data analysis as mentioned in [13].

Ethical considerations:

The local Ethics Committee of University of Baghdad approved the conduction of the study (CSEC/0122/0002). Written informed consent was taken from all participants. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

The collected data were coded, processed and analyzed using the SPSS (Statistical Package for Social Sciences) version 20 for Windows® (IBM SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) and Fisher's exact test to calculate difference between two or more groups of qualitative variables. Quantitative data were expressed as mean and standard deviation (SD). Independent samples t-test was used to compare between two independent groups of normally distributed variables (parametric data). To evaluate the best cut-off points of miRNA-125b for predicting analysis of the receiver operating characteristic (ROC) curve was done. To assess the test's accuracy, the area in the curve line (AUC) value was computed. P value ≤ 0.05 was considered significant.

RESULTS

Table 6 summarizes the patients and controls clinical and demographic characteristics.

Table 6: Baseline characteristics of ankylosing spondylitis patients and healthy controls

Characteristics	AS Patients (n =100)	Controls (n = 100)	P-value
Age (year)	40.56 ± 10.19	38.78 ± 10.57	0.227 NS
Disease duration (year)	9.04 ± 2.03	-	-
HLA-B27 positive	57%	-	-
BASDAI	3.45 ± 2.03	-	-
BASFI	3.84 ± 2.62	-	-
ESR (mm/hour)	25.39 ± 19.59	-	-
CRP (mg/L)	18.34 ± 3.45	-	-

miRNA-125b expression levels in AS patients

The quantitative expression of miRNA-125b was transcription quantitative polymerase chain reaction reverse was used to evaluate (RT-qPCR) and the relative quantification method. Normalization of gene expression "measured by the folding (2-Ct) method and quantified to the level of a housekeeping gene (miRU6 gene)". A representative RT-qPCR plot is given in **Figures 1 and 2**.

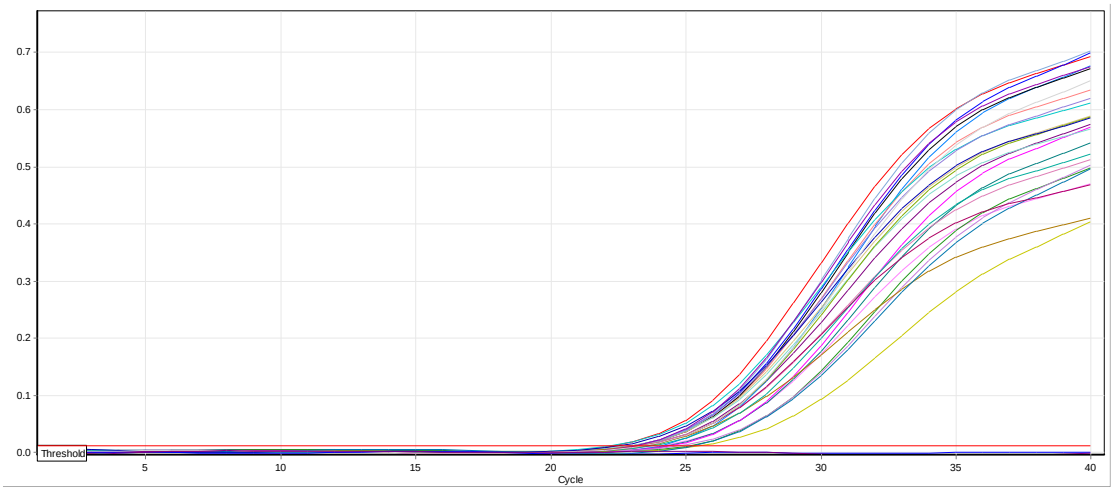

Figure 1: miRNA-125b qPCR amplification graphs. Samples came from every group of the study. Image was captured straight from a Qiagen Rotor gene qPCR machine.

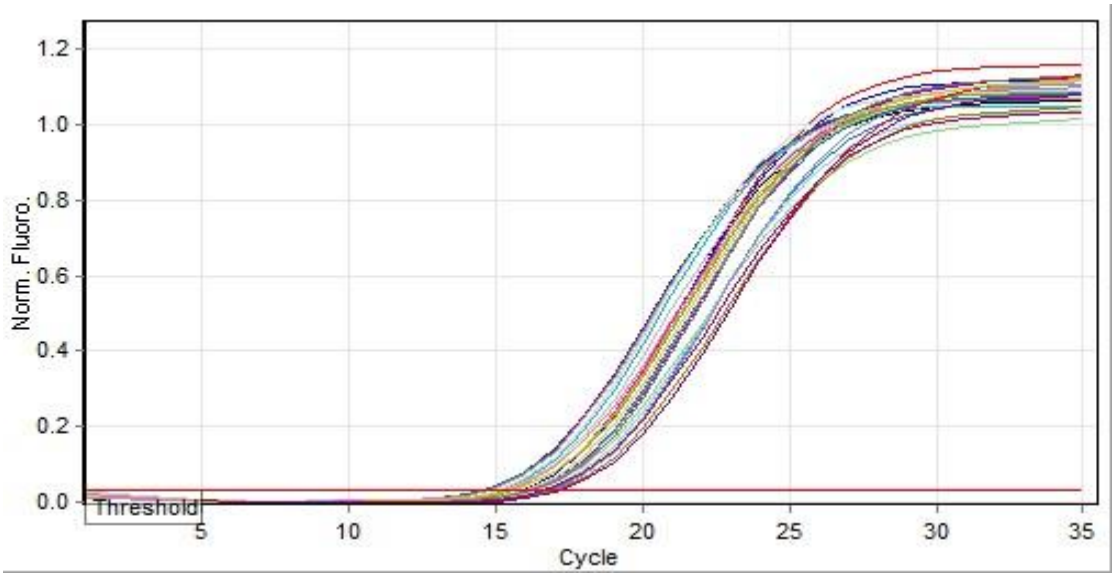

Figure 2: miRU6 (housekeeping gene) qPCR amplification graphs the samples included every research group. The image was captured straight from a Qiagen Rotor gene qPCR equipment.

Figure 3 depicts a typical melt curve for the miRNA gene for materials examined by RT-PCR. The amplicons are displaying a single peak. These results are explained by the fact that for each sample, the melt curve reflected a single, pure amplicon, and the intercalating dye test was thought to have excellent specificity for amplification.

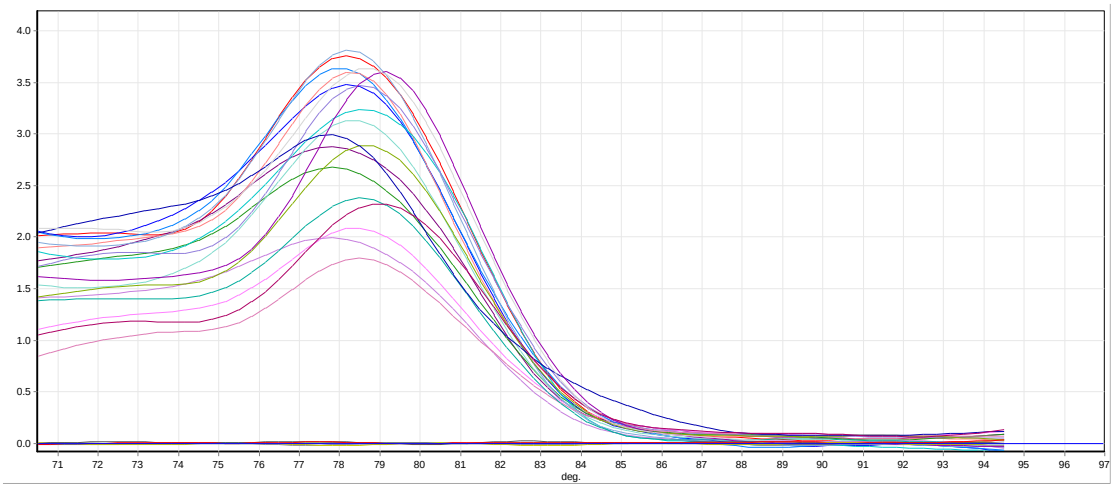

Figure 3: Melt curve of miRNA-125b gene amplicons after RT-qPCR analysis showing single peaks.

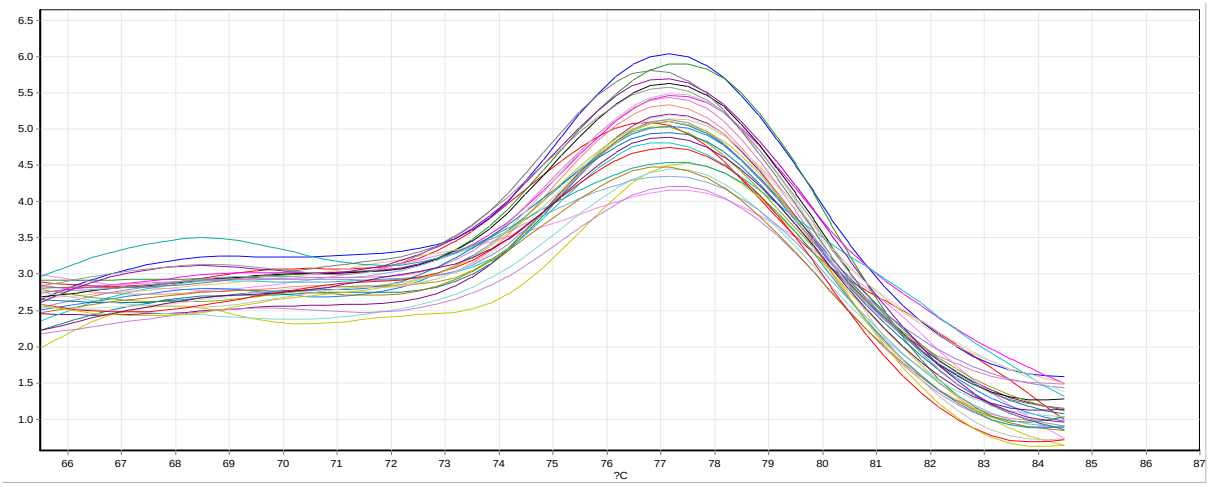

Figure 4: Melt curve of miRNA- U6 gene amplicons after RT-qPCR analysis showing single peaks.

The ΔCt mean of miRNA-125b gene was increased in AS patients (10.61 ± 1.61) compared to the corresponding ΔCt means in controls (**7.68** ± 0.94), but the differences were not significant (p<0.0001), **table 7**.

However, the relative expression ($2^{-\Delta\Delta Ct}$) of miRNA-125b was decreased by 0.133 folds in AS patients.

Table 7. Expression level (ΔCT) of miRNA-125b in AS **patients and control groups**

Gene	Control (No.=100) ΔCT (mean ± SD)	AS Patient (No.=100) ΔCT (mean ± SD)	*P-value*
miRNA-125b	7.68 ± 0.94	10.61 ± 1.61	<0.0001**

Receiver operating characteristic (ROC) curve

A Receiver Operating Characteristic curve analysis was used to evaluate the diagnostic performance of miRNA-125b in distinguishing people with AS from control ones. With a sensitivity and specificity of 92% and 91%, respectively, the analysis revealed that miRNA-125b at an ideal cut-off point of 8.94 could significantly discriminate patients from controls (AUC=0.962; 95% CI: 0.9398-0.9854, p 0.0001). The cut-off point was adjusted with Youden index (YI=0.38).

This ROC curve generated for miRNA–125b expression "to calculate their predictive performance as an AS acute phase marker. ROC curves were above the diagonal and showed good sensitivity and specificity" (**Figure 4**).

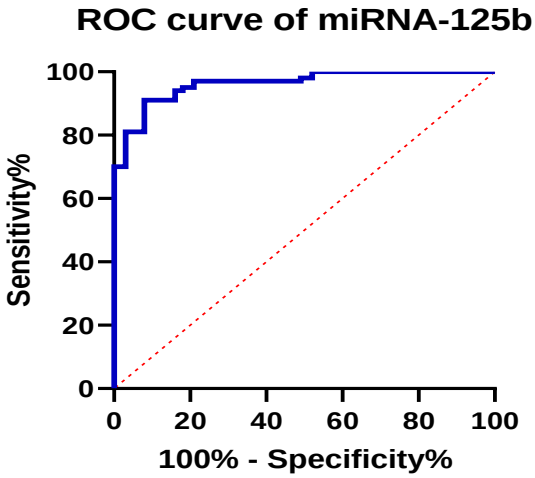

Figure 4: Analysis the miRNA-125b receiver operating characteristic (ROC) curve for identifying Ankylosing Spondylitis (AS) patients from healthy controls.

DISSCUTION

Numerous researches imply that genetic and environmental factors contribute to AS propensity and susceptibility [(14)].

AS a perfect disease example correlation with a genetic marker because it has been linked to HLA-B27 (human leukocyte antigen B27) for a long time [(14,15)]. 90% of people with AS express HLA-B27, but is only in less than 8% of the general sample [(16,17)]. However, the role of pathogenetic of this gene not yet sufficiently elucidated. Several theories have been put out to clarify how HLA-B27 can cause AS: (1) According to the arthritogenic peptide theory, AS may be brought on by cytotoxic T cell lines that due to molecular mimicry between infections and self-peptides, they cross-react with each other [(18)]; (2) The

endoplasmic reticulum (ER) accumulates misfolded HLA-B27, which activates signaling cascade in the intracellular and causes ER-related stress with unfolded response of protein, according to the HLA-B27 misfolding hypothesis [19]; (3) In accordance with the theory linking the creation of HLA-B27 homodimer on the cell surface to an inappropriate immune response, these dimers are recognized by killer immunoglobulin-like receptors, which are expressed on the exterior of natural killer and CD4+ T cells [20] . The Delgado's functional index and the Bath Ankylosing Spondylitis Functional Index are the two functional indices that are most frequently used to evaluate functional capacity [21]. The 10 questions on the BASFI are answered using a VAS. The average of the questions' final scores—which range from 0 (no restriction) to 10-determines the grade (maximal limitation in function). It has been demonstrated that both functional indices can distinguish across patient groups with varying degrees of physical function improvement.

The two disease activity markers that are most frequently used in clinical research and practice are two functional indices, CRP and ESR. However, they lack appropriate sensitive and specific [22] and do not adequately represent the process of disease in AS [23]. Both the ASDAS, a gauge of disease activity, and the ASAS AxSpA classification criteria entail an elevated CRP [21].

On the other hand, only 40–50% of AS patients show elevated CRP or ESR [24]. As a result, a normal ESR or CRP does not completely eliminate AS or reflect current illness. Compared to patients with non-radiographic axial SpA, those with AS showed increased concentrations of the both acute-phase reactants [25]. Additionally, improved spine X-ray radiographic alterations are linked to elevated CRP [24]. MRI signs of inflammation at the sacroiliac joint [26]. Additionally, higher CRP and ESR levels in AS patients suggest sacroiliac joint and spine radiographic degeneration in the future, According to recent studies, in patients with axial SpA, hsCRP correlates more favorably than CRP with clinical disease activity markers [27]. Additionally, According to the RT-qPCR results, the expression of miRNA-125b was significantly lower in AS patients than that in healthy control (p-value <0.0001). This result was agreed with **Tan *et al***. [1] who discovered that compared to healthy controls, miR-125b-5p expression was significantly reduced in AS patients (HCs). The miR-125 family includes the miR-125a, miR-125b-1, and miR-125b-2 molecules [28]. The NF-κB signaling pathway can be adversely regulated by miR-125a and miR-125b by acting on TNFAIP3, which offers a fresh perspective on how immune cells are activated [29].

A growing body of research has linked autoimmune diseases to miR-125. According to reports, donors with active AS exhibit higher levels of miR-125a-5p expression while experiencing lower levels of miR-125b-5p expression **Hruskova *et al.*** and **Perez-Sanchez *et al.*** [30,31], which is partly incompatible with our results. In this study, the miR-125b expression in whole blood cells of patients with primary AS was significantly lower than that in HCs. This might be connected to the study participants' varied racial backgrounds. Therefore, we proposed that the miR-125 family may control the etiology of AS by modulating immunological signaling or osteogenic differentiation pathways. Therefore, additional research into the precise mechanism by which miR-125 controls AS is necessary.

In conclusion, AS patients have lower levels of miRNA-125b expression than do healthy controls. The findings imply that miRNA-125b's low expression in AS patients may act as a biomarker for diagnosis.

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- **Competing interests:** None.
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- **Conflicts of Interest:** authors declare that they have no conflicts of interest with regard to publication of this paper.

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