Iraqi Journal of Biotechnology, May 2019, Vol. 18, No. 2, 242-251

Association between Ankylosing Spondylitis and the miR-146a Polymorphisms a Samples of Iraqi Patients

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Received: February 17, 2019 / **Accepted:** April 10, 2019

Abstract: MicroRNAs (miRNAs) are small noncoding sequence that regulate the expression of multiple target genes at the post-transcriptional level, efficiently regulating fundamental cellular processes such as proliferation, apoptosis, and development. Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic illness of mysterious etiology with a strong genetic susceptibility. Important development is being made in our considerate of the pathogenetic tools involved in this illness, and the recent genome-wide association study (GWAS) consequences have related at least 60 loci to As, The present study explored the association between ankylosing spondylitis (AS) and two single nucleotide polymorphisms (SNPs), miR-146a rs2910164G>C, in Iraq population. The genetic analysis of the Single Nucleotide Polymorphisms, for miR-146a gene by using PCR-SSCP technique that there was significant difference in genotype polymorphisms between patients and control . Also this research found relationship between miR-146a and Osteoprotegerin ,interleukin 23serum level in AS patients .

Keywords: miRNA-146 , polymorphisms , PCR-SSCP, AS .

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Introduction:

Ankylosing spondylitis is a public inflammatory illness which often disturbs the function of axial joints and peripheral joints. Patients commonly undergo early indications of back pain and arthritis for numerous year(1), While exercise can progress the joint functions, rest may have opposite effect. Previous studies suggest that spondyloarthropathies was described by initial enthesitis and synoviti(2).

MicroRNAs recently received great attention as important genomic regulators in different pathways such as differentiation of hemopoietic cells, proliferation of cells ,developments of organs and apoptosis .MicroRNAs are small (21-23 nucleotides in length) endogenous RNAs, and non-coding but functional ,via their binding to 3'UTR (Un-Translated Region) to specific mRNA targets lead to negative regulation of gene expression and this lead to believed that it may participate in immune function, apoptosis and cell differentiation. Other studies(3,4) shown that microRNAs may have correlation with pathological changes of AS found that patients with AS compared to controls had significantly higher levels of miR-21, PDCD4 mRNA, and CTX .MicroRNAs (miRNAs, miRs) are a group of endogenous, 20–25 nucleotides long non-coding RNAs. Utmost miRNAs are transcribed by RNA polymerase II, and

their upstream controlling regions comprise canonical core promoters and enhancers, controlled by transcription factor(5). They purpose as posttranscriptional regulators of gene expression by exactly interacting with definite mRNAs and inducing their degradation or suppressing their translatio(6).

Agreeing to the miRBase database and other bioinformatic data, a developed miRNA can fix to many mRNA targets, and at least one-third of human protein-encoding genes seem to be controlled by miRNA. Thus, miRNAs have been associated in a wide variety of biological processes, including cell growth, differentiation, proliferation and apoptosis Over the past numerous years, it has become progressively clear that miRNAs are not only significant for usual organismal improvement and physiology but also in the pathologies of autoimmune illnesses, cancer, heart illness, and inflammation(7). Concerning autoimmune illnesses, it has been described that numerous have similar fundamental etiology and have shared susceptible gene(8).

miR-146a is amongst the most studied miRNAs and is encoded by chromosome 5q33. Mature miR-146a can bind to the 3'-untranslated regions of many target mRNAs, including interleukin-1 receptor-associated kinase 1 (IRAK-1), IRAK-2, tumor necrosis factor receptor-associated factor 6 (TRAF-6) and additional transcripts related with inflammatory signalin(9,10). It has been anticipated that miR-146a contributes in Toll-like receptor and cytokine signalin(11), thus adaptable the immune response. Accumulating confirmation also advocates that miR-146a can be induced by NF-κ(12). Single nucleotide polymorphisms (SNPs) are known to be the most common type of genetic variant in the human genome. SNPs situated in miRNA regions can change miRNA expression and/or maturing to disturb function in three ways: through the transcription of the primary transcript, through pri-miRNA and pre-miRNA processing, and by affecting miRNA–mRNA interface(13).

Lately, much exertion has been made in the direction of studying the role of SNPs in miR-146a and miR-499 and how these miRNAs may affect the normal actions of cells and the pathogenesis of many illnesses. The communal miR-146a polymorphism rs2910164 involves a G>C nucleotide replacement. It can clue to the alteration from a G:U pair to a C:U mismatch in the stem structure of the miR-146a precurso(7). It has been described that miRNA expression could be changed in synovia, peripheral blood mononuclear cells (PBMCs) or T cells from patients with different forms of arthritis, including RA, OA and AS(8).

Case-control studies revealed that MIR146A gene SNPs increased susceptibility in the onset of several autoimmune diseases. Positive results were obtained in systemic lupus erythematosus (SLE), psoriatic arthritis (PsA), asthma and telangiectasia in systemic sclerosis(14,15,16,17). And a recent report mentioned the association between MIR146A SNP rs2910164 and ankylosing spondylitis (AS) in Chinese subjects(16). In another hand, there were also conflicting negative association reports in PsA, RA and SLE(15) .

Subjects Material and Methods:

Study Design, Setting and Data Collection Time:

Case-control study was conducted between March2016- July 2016 and it was carried out at the Rheumatology unit / in Babylon province/Iraq .

Study Population:

The study subjects comprised from 70 patients suffer from AS fulfilling the modified New York criteria for classification, and these patient under biological therapy randomly selected from mrjan teaching hospital (67 male and 3 female) as AS patients group with age average (20-60 year), the control group study included 40 people apparently healthy that included (38 male and 2 female) with age average (20-60 year),this control group matched with patient group. All subjects in this study were taken written consent before participation in this study.

Exclusion Criteria:

The excluded include patients Diabetic mellitus, hypertension, hepatitis, heart failure, renal failure, liver disease, malignant disease ,patients on chemotherapy, etc. and excluded patients who suffer from complication AS. Questionnaire taken from the patient included : age, sex ,occupation , smoking habit, alcohol intake, and family history, past medical history, BASDI,BASFI and type of Biological therapy.

Materials and methods:

1. DNA was extracted from freezing blood according to kit leaflet (genaid, genomic DNA extraction kit).
2. Primers and PCR conditions; primers were used in present study are shown in (Table 1) (18), PCR conditions are shown in (Table 2).
3. ata analysis, the statics analysis implemented using Qi square and odd ration at p value <0.05.
4. Haplotype frequency were determination by variety of bands between patients and control. While the hormone OPG, IL-23, TNF and SOS according to kit leaflet (Biomerieux (France).

Table (1): The sequence of (miR-146a rs2910164 C-G) primers.

Descriptive	Sequence	Size product
Sense	F 5-GCTACGTGGACGACACGCT-3	195bp
Antisense	R1 5-CTCGGTCAGTCTGTGCCTT-3	

Table (2): The program used for *miR-146a* amplification sequence.

Stage	Temp.(C°)	Time(min)	Function	Cycles
1	94	5:0	Initial denaturation	
2	94	0:30	denaturation	30
	57.8	0.20	Primer annealing	
	72	0.50	Template elongation	
3	72	10	Final elongation	
4	4	5	Incubation	Hold

Result:

The association of study groups (Patient Vs Controls):

This study showed that the majority of patients with AS were male (96%) , (53%) came from Babylon province and (60%) of them were not employed, There was significant association between patients and control regarding the occupation (OR 0.4**)** , presence of family history for AS (OR 10.5) , meanwhile there was no significant association between patients and control with regarding gender, residence , type treatment as shown in Table (3).

Table (3): The association of study groups by study variables.

Variable	patients No. (%)	Control No. (%)	Odds ratio	95%CI	P-value
Gender					
Male	67 (96%)	38 (95%)			
Female	3 (4%)	2 (5%)	1.1754	0.188 to 7.349	0.86
Residence					
Babylon	37 (53%)	40 (100%)			
Karblaa	11 (16%)	0 (0%)			
Najaf	15 (21%)	0 (0%)			
Al-qadisiyya	7 (10%)	0 (0%)			
Occupation					
Employee	28(40%)	25(62.5%)			
Not employed	42(60%)	15(37.5%)	0.400	0.179 to 0.889	0.024*
Family history					
Present	25 (36%)	2 (0%)			
Absent	45 (64%)	38(0%)	10.55	2.34 to 47.47	0.002*
Type treatment					
infliximab	`58(83%)	0 (0%)	4.68	0.08 to 247.29	0.44
Enbral	12(17%)	0 (0%)			
Smoker					
yes	29(41%)	17 (57.5%)	0.957	0.435 to 2.102	
No	41 (59%)	23 (42.5%)			0.912

*** t-test at P ≤ 0.05.**

The distribution of the study population by Body mass index:

The results showed that the majority of patients were pre-obese (33%), while (12 %) of them were obese and (22%) were normal .In the control group the majority were normal (12%), (16%) were pre-obese and (12%) were obese, as shows in (Figure 1).

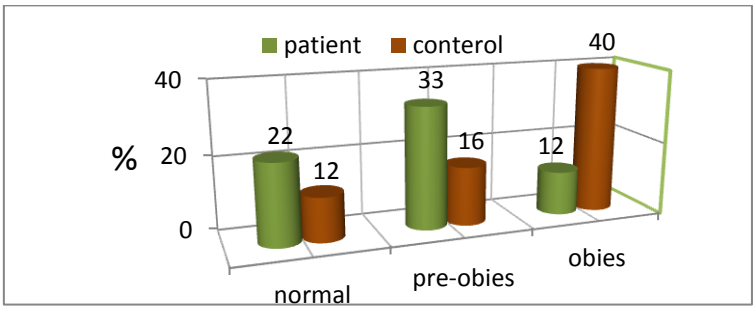

Figure (1): Distribution of study population by BMI (kg/m^2).

The differences between age and BMI for both genders in patients and control:

This study showed significant mean differences of age between patients and the control groups and there no significant mean differences of age for both male and female, meanwhile there were significant mean differences in body mass index between patients and control group but no significant mean differences between male and female, as shown in (Table 4).

Table (4): Mean differences of age and BMI for both genders in Ankylosing Spondylitis patients and control.

Group Indicates	Control Mean ± SD		Ankylosing Spondylitis Mean ± SD		P value of gender	P value of group
	Male	Female	Male	Female		
Age (years)	5.89±1.32	4.00±1.73	2.38±0.96	3.00±1.00	0.92	0.001*
BMI kg/(m^2)	4.91±0.75	4.66±1.15	2.02±0.71	2.66±0.57	0.33	0.001*

*** t-test at P ≤ 0.05**

Molecular Results:

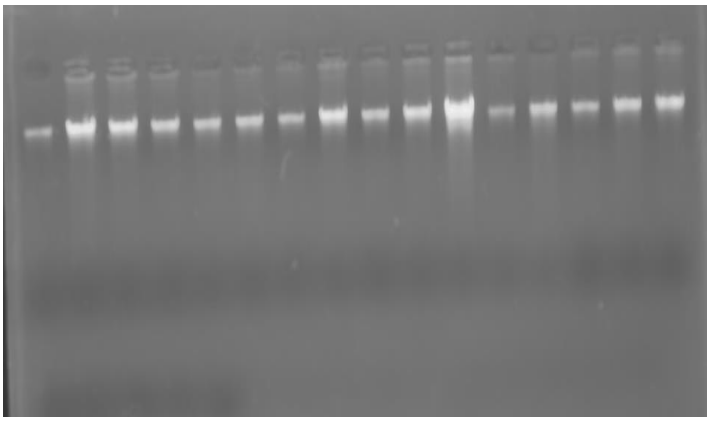

Figure (2): The electrophoresis pattern of DNA extracted from blood for patients AS and control, 1% agarose, 75 V, 20 Am for 1h. (10 µl in each well).

MIR-146a genotyping:

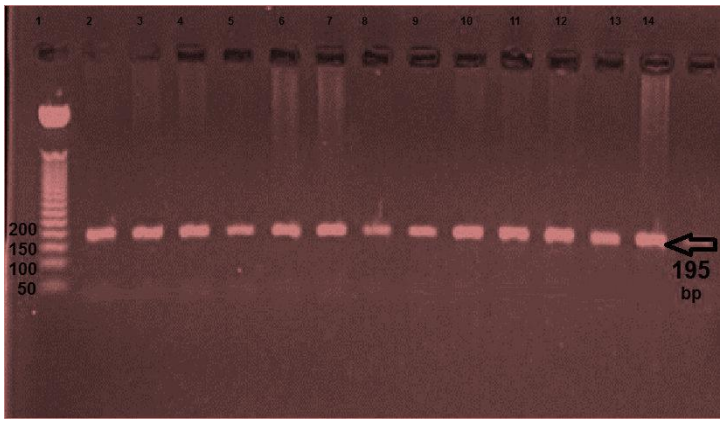

Figure (3): The electrophoresis pattern of PCR product for MIR-146a gene ,this amplification product one band 195 bp for both patients and control ,1% agarose ,75V,20Am for 1h. Line 1 DNA marker (1500 bp), line 2-8 PCR product of patient, lane 9-114 PCR products of control.

PCR-SSCP MIR-146a gene:

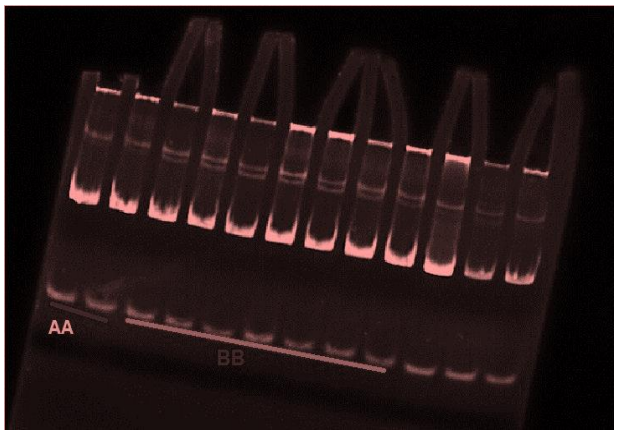

Figure (4): The electrophoresis, pattern of PCR-SSCP technique for MIR-146a gene (Pattern A:3 bands) and (Pattern B:4 bands) for both patients and control. Electrophoresis conditions: polyacrylamide gel concentration 8%, power applied: 85V-100mA, time to run: 120 min .staining method ethidium bromide.

The pattern B were more frequent in the patients with AS than control group with odd ratio (6.151) as shown in (Table 5).

Table (5): The genotype distribution and odd ratio of MIR-146a gene polymorphism for patients and control.

Pattern name	Patients AS%	Control%	P- value	Odd ratio	95 % CI
BB	49(70%)	11(27.5)%	< 0.0001	6.151	2.5979 to 14.5661
AA	21(30 %)	29(72.5%)			

Role of MIR-146a physiological Biomarker levels rate in Ankylosing Spondylitis patients and control subjects:

The statistical analysis show the presence of significant differences ($p \leq 0.05$) in Osteoprotegerin and Interlukin-23 in patients with AS who have pattern B when compared with patients who have pattern A. where no significant differences in Sclerostin and Tumor necrosis factor TNFα in patients with AS who have pattern B when compared with patients who have pattern A.

In the control group, the statistical analysis indicate there were no significant differences in levels rate in control subjects who have pattern A when compared with subjects who have pattern B. on other hand The statistical analysis of this study shows the no significant differences ($p \leq 0.05$) between patients and control groups in all parameters According to pattern of MIR-146a genotype, as shown in (Table 6).

Table (6): Role of MIR-146a and physiological parameters levels rate in Ankylosing Spondylitis patients and control subjects.

Parameters	P value of group		
	Control Mean ± SD	AS patients Mean ± SD	
Osteoprotegerin (µg/ml) (A)	7.10 ±1.89	8.01± 2.54	0.7
(B)	8.12± 2.91	11.91±4.58	0.6
P value Of group	0.3	0.00002	
Interlukin-23 (µg/ml) (A)	28.97± 4.49	37.08 ± 11.31	0.5
(B)	29.67± 3.15	45.63± 16.99	0.6
P value Of group	0.5	0.01	
Sclerostin(µg/ml) (A)	3005.6 ± 1425.8	3092.21 ±1708.9	0.9
(B)	3050.43 ±1134.30	3301.06 ±1650.4	0.9
P value Of group	0.9	0.61	
Tumor necrosis factor TNFα (µg/ml) (A)	37.44 ±14.02	46.89± 28.814	0.7
(B)	46.03 ±14.45	50.64± 31.02	0.9
P value Of group	0.1	0.6	

*** t-test at P ≤ 0.05 , (Mean ± SD): Mean± Standard Deviation**

Discussion:

MicroRNA considers as regulatory factor which have starring role in gene expression regulation, moreover the review of texts about microRNA levels and its genetic data were little. Nevertheless, the role of microRNA was considered in cancer in more researchers, there is a insufficient studies about its genotyping and genotyping role in physiological considerations, as a consequences of these reasons the current study was recommended to study genotyping of microRNA and its role in some physiological parameters in communal illness, AS patient. Four microRNA genes were selected in current study miR-146a.

The consequences display there were difference between patients and control genotyping and microRNA have role in some physiological parameters as display underneath. The amplification of miR-146a (rs2910164 C-G) gene by PCR show195 bp, as show in (Figure 4).

The by means of of PCR-SSCP products size technique in current study were selected in current study because these techniques are fast and low cost equate with other techniques, and it give an sign about polymorphisms of genes, so it acknowledged and dependent in researches, the current study is the first study in Iraq and its one of the imperative research in the world that covenant with microRNA gene polymorphism and its polymorphism role in AS patient. The consequences of gene polymorphism of miR-146a displayed that the pattern B was more common in AS patients than control as revealed in (Table 6). The study of miRNA biology has involved cumulative attention, subsequent in rapid progresses in this area. SNPs in the miRNA gene area may straight disturb the expression of the mature miRNA, subsequent in varied functional consequences. To period, numerous epidemiological revisions have showed relations between SNPs in miRNAs and cancer susceptibility(6), autoimmune diseases(18), schizophrenia(19) and cardiovascular illness(20). we establish

that the miR-146a rs2910164 variation was concomitant with enlarged danger of AS at both the genotypic and allelic levels and These snp in this study covenant with with reported manipulating MIR146A gene expression(21,22). Even though (rs2910164) was(16) reported as positive with AS. SNPs rs2910164 C allele reduced the amount of pre- and mature miR146A 1.9- and 1.8-fold, correspondingly(23). Covenant Occurrence of this Pre-RNA allele in Chinese (58.9% in this study) is much greater than that in Europeans (for example 27.1%)(24). The influence to autoimmune diseases of this functional microRNA gene SNP may be somehow dissimilar between populations.

In broad-spectrum, sequence differences in miRNA genes, comprising pri-miRNAs، pre-miRNAs and matured miRNAs, have the possible of manipulating the handling and/or target selection of miRNAs. As a result of SNPs present in pri-, pre- and mature-miRNA, abnormal expression of hundreds of genes and pathways, importantly disturbing miRNA function, may happen(25) and this may illuminate the unjustified alteration in the levels in some hormones and physiological parameters in this study. There are three choices of intrusive with miRNA function on the foundation of SNPs in miRNA sequences: (1) changes in the pri- or pre-miRNA may disturb their constancy or treating efficiency; (2) alterations performing in cis or trans on the pri-miRNA promoter may stimulus the transcription rate and (3) a sequence of the mat-miRNA might be changed، thereby steadying or disrupting its communication with mRNA targets؛ SNPs in mat-miRNA sequences may be further sub-classified into the subsequent two sub-categories: (i) SNPs within the miRNA 5--seed region, from positions 2-to 7, which are accountable for specificity of target recognition and (ii) SNPs within miRNA 3--mismatch tolerant region that is talented of tolerating mismatches to a certain extent(23).

Agreement with Other study Xu *et al.* (2015) (16) discovered the relationship between AS and 2 SNPs, miR-146a rs2910164 and miR-499 rs3746444, in a Han Chinese population. A case–control study comprising of 102 subjects with AS and 105 healthy controls was considered. They found that there is a significant alteration in the miR-146a rs2910164 SNP. This communal polymorphism, rs2910164 G/C in miR-146a, variant in this Snp arrangement with Other Study For two causes, many people hold the view that the G allele confers a higher expression level of the mature miR-146a: 1) The change from C to G may increase the stability of the miR-146a. One study showed that the optimal free energy of miR-146a differed between people with the G allele or the C allele(26). The unrestricted energy of the paired strand with the G allele was determined to be—26.8 kcal/mol, while that of the C allele was-24.0 kcal/mol. This shows that this SNP may disturb the constancy of the miRNA, thereby manipulating its expression level 2)(27). The G allele showed amplified production of mature miR-146a equated with the C allele because the C allele was institute to have lower transcriptional activity and weaken the processing of pri-miR-146a(28).

Other study demonstration Concentrated investigations of miRNAs known to control these pathways may prime to further detection of novel AS-

related miRNAs. Prominently, the function and downstream molecular pathways of numerous key decontrolled miRNAs, such as miR-16, miR-221, and miR-29a, persist to be explained. Dysregulation of these miRNAs has been established with a rationally large sample size(29).

Two acknowledged gene targets of miR-146a are the TNF receptora ssociated factor 6 (TRAF6) and the interleukin-1 receptor-associated kinase 1 (IRAK1) ,Even though no significant alteration in the mRNA or protein levels of TRAF6 or IRAK1 were perceived between RA patients and control subjects, the suppression of TRAF6 and/or IRAK-1 in THP-1 cells caused in TNFα reduced levels , Consequently, it seems that the up-regulation of miR-146a expression may consequences in lengthy TNFα production through the derestricted expression levels of TRAF6/IRAK1, which are key device molecules downstream of the Toll-like and cytokine receptor signalling pathway(30). In adding, IRAK2, FADD, IRF-5, Stat-1, PTC1, FAF1 genes were also recommended as mir-146a targets, a miRNA linked to inflammation and apoptosis progressions(31).

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