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H19 Single Nucleotide Polymorphism (rs2251375): A Predictor of Increased Resting Heart Rate in RA Egyptian Patients: A Pilot Study

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ABSTRACT:

Background: Rheumatoid arthritis (RA) is a systemic autoimmune chronic inflammatory disease that could lead to multiple morbidities and mortalities with cardiovascular diseases being the commonest. In search for possible new therapeutic targets, long non coding RNA genes came into interest, particularly H19 which was proven to be a positive regulator of inflammation and a possible predictor of complications.

Aim: To investigate the association between H19 SNP (rs2251375) genetic variants and clinical features of RA patients.

Materials and Methods: 100 RA patients (74 females and 26 males) were recruited in this study. Clinical examination was done including heart rate and blood pressure measurements. RA-related laboratory investigations were done and patients' clinical data was recorded. Real-time PCR genotyping of (rs2251375) was done using TaqMan® MGB probes.

Results: The heterozygous genotype CA was the most prevalent among the studied group (55%). There was a statistically significant association between the genotype CA and increased resting heart rate (p=0.04) and ESR (p=0.04). No association between (rs2251375) and blood pressure, CBC parameters, CDAI, RADAI-5 or HAQ (p>0.05).

Conclusion: There is a significant association between H19 SNP (rs2251375) and increased resting heart rate and ESR, but there was no statistically significant association with other clinical and laboratory RA features.

Introduction:

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that has an inclination to affect females more frequently than males with a ratio of 1.3:1 to 12.5:1, being predominantly observed in the elderly. It's a multifactorial disease with a wide range of co-morbidities and co-mortalities. Cardiovascular complications are one of the most common in RA. The international prevalence of RA is estimated to range from 0.24 to 1% and in Egypt it is estimated to be 0.2-0.3**%** (1–3)**.**

For decades, RA has been thoroughly studied to draw the outlines of its pathogenesis and major risk factors. However, due to the disease convoluted nature, it has become a challenge to name all the possible etiological aspects. Nonetheless, genetic and epigenetic factors had been a primary research area for RA pathogenesis and possible new therapeutic strategies.

Long none coding RNAs (LncRNAs) had been a very rich area of research in substantially all diseases. LncRNAs, which are a type of RNA that has more than 200 nucleotides, were primarily marked to be nonfunctional RNAs. However, they were discovered to be capable of controlling gene expression, including gene transcription, RNA splicing, chromosomal remodeling, and protein transport. Furthermore, lncRNAs can interact with proteins to affect their functions and can associate with RNA molecules to regulate their translation, such as mRNA and miRNA (4,5)**.**

H19 was one of the early discovered lncRNAs. Its gene is only expressed from the maternally inherited chromosome 11p15.5. H19 gene's location is quite unique as it exists in an imprinted region of chromosome 11 close to the insulin like growth factor 2 (IGF2) gene locus (6)**.**

The expression of H19 transcripts was shown to be higher in RA synovial tissue than in osteoarthritic patients, subjects with joint trauma, and normal controls both in situ and by semi-quantitative PCR. H19 has the capacity to control the expression of genes involved in classical inflammatory pathways such as NF-κB, p38/MAPK/mTOR (mammilian target of rapamycin), toll-like receptor, and TNF-α. Consequently, H19 is considered an inflammatory positive regulator (7–9)**.**

Interestingly, H19 overexpression in cardiac muscles results in marked dilatation of the cardiac chambers with ensuing fibrosis and up-regulation of multiple extracellular matrix-modifying genes (10). Cardiovascular disease (CVD) is one of the most common complications in RA patients. Development of CVD in RA patients depends on two important factors: vascular damage and endothelial dysregulation, both of which show an association with H19 expression in cardiac muscles (10,11).

Rs2251375 polymorphism is one among the commonest SNPs for H19 gene, as it has the highest minor allele frequency (MAF = 0.48) in the human population. Rs2251375 is located at the 5′ region of the H19 gene along others such as (rs2067051 and rs4929984) in the position chr11:1998266 (GRCh38.p13 with alleles C>A/C>T). It is an intron variable (12,13).

Taking into consideration the established role of H19 gene expression upregulation in RA patients, we took interest into studying the possible role of its single nucleotide polymorphism (rs2251375) in clinical features and predictors of complications in RA patients.

Materials and Methods:

Study Population:

One hundred RA patients were recruited for this cross-sectional study, including seventy-four female and twenty-six male patients, all of which diagnosed according to the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria (14)**.** All the study patients were enlisted as study subjects from Suez Canal University Hospital.

To limit the confounding factors effect, patients with other autoimmune or inflammatory diseases were excluded from the study. A written consent had been collected from the participants.

Personal and medical histories of the patients were collected. Also, their clinical features such as heart rate, blood pressure, disease duration and number of tender and swollen joints were recorded. RA disease activity was assessed using the Disease Activity Score 28 (DAS28), Clinical Disease Activity Index (CDAI) and Rheumatoid Arthritis Disease Activity Index-5 (RADAI-5) (15). Patients had provided a written consent which was approved by the Ethical Committee in Faculty of Medicine in Suez Canal University (research3772#).

Laboratory Investigations:

Complete blood count (CBC) was analyzed using Sysmex XN-1000 hematological analyzer. RA laboratory investigations and markers of inflammation were both done for the patients. Erythrocyte sedimentation rate (ESR) using the Westergren method. All patients have been genotyped for H19 SNP (rs2251375).

H19 SNP (rs2251375) C>A

- **DNA Extraction:**

The DNA was extracted by spin-column technique kit from whole blood (PureLink® Genomic DNA Mini Kit) according to the provided protocol by Life Technologies (Thermo Fisher Scientific Inc. USA). The purity of the extracted DNA and its yield both were assessed by using the Thermo Scientific NanoDrop™ Spectrophotometer (Nanodrop) (Thermo Fisher Scientific Inc. USA).

- **Amplification of Genomic DNA:**

Amplification of the extracted DNA was done using StepOne™ Real-Time PCR system. The PCR mixture was a total of 20 µl solution that encompassed a 10 µl of Master Mix (Thermo Fisher Scientific, USA), 4.5 µL RNAse free water, and finally 0.5 µL of the sample extracted DNA and same amount from the TaqMan assay mix of genotyping.

Two TaqMan® MGB probes (Thermo Fisher Scientific, USA) with diverse dyes **(VIC®/FAM™)** were used for the sequence-specific forward and reverse primers to amplify the following polymorphic sequence:

H19 SNP (rs2251375):

CTTCCTGCCACCATCACGGCTCAGA**[A/C]**CTCACGTTCCTGGAGAGTAGGGGTG

Each run had a negative control for validation. The PCR reaction encompassed an initial denaturation at 95°C for 10 minutes which was immediately followed by a total of 40 cycles of 15 seconds at 92°C and 1 minute at 60°C. PCR results were later analyzed using StepOne™ Real-Time PCR system software (Thermo Fisher Scientific, USA). Allelic discrimination was used for the analysis.

Statistical Analysis:

Data were processed using SPSS version 26 (SPSS Inc., Chicago, IL, USA). Mean and standard deviation were used for quantitative data representation. Numbers and percentages were used for all the qualitative data. To measure statistical significance between variables, ANOVA and Student t test were

used. For the analysis of our qualitative data, we used Chi-square and Fisher Exact test. Finally, a probability value (p-value) <0.05 was considered statistically significant.

Results:

The study included 74 female and 26 male RA patients with mean age of 45.82 ± 9.8 years. Genotyping H19 SNP (rs2251375) revealed that the heterozygous genotype CA was the most prevalent among the studied group (55%) **(Figure 1)**. The C allele proved to be the most frequent as well in the patients (58%).

Running an analysis of patients' clinical features **(Table 1)**, we found a significant statistical difference between increased heart rate and the genotype CA (p=0.04) with a mean of 90.5 beat/min **(Figure 2)**. Similarly, there was a significant association with the allele C (p=0.012). However, there was no association with blood pressure, pulse pressure or CBC parameters (p>0.05).

There was a statistically significant association between (rs2251375) and high ESR with a p-value = 0.04 with the CA genotype**.** However, there was non-statistical significant association with Clinical Disease Activity Index (CDAI), Rheumatoid Arthritis Disease Activity Index-5 (RADAI-5) or Health Assessment Questionnaire (HAQ) (p>0.05) **(Table 2).**

DAS28ESR Ranks distribution according to H19 genotypes show a correspondence between severe ranks and CA genotype **(Figure 3).**

Running a regression analysis for heart rate and ESR against (rs2251375) heterozygous CA genotype proved that those two parameters can be used as inflammatory predictors **(Table 3).**

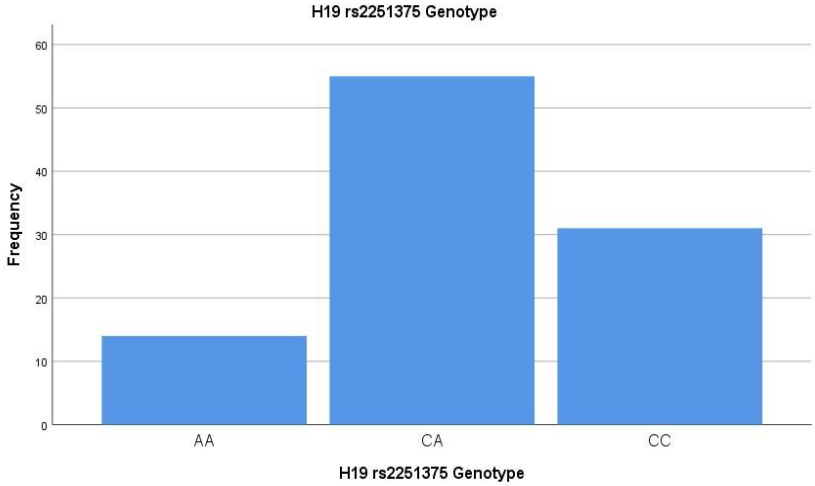

Figure (1): H19 SNP (rs2251375) Genotypes and Alleles Distribution in RA patients

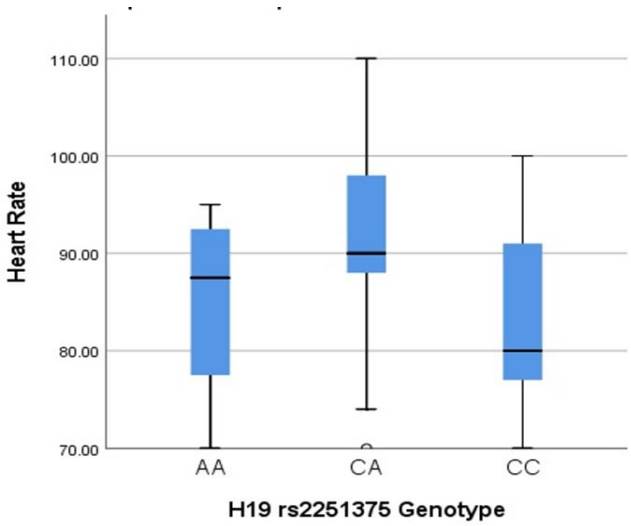

Figure (2): Heart Rate Measurements According to H19 SNP (rs2251375) Genotypes

Table (1): Clinical and laboratory Data of RA Patients

Variables	Mean	Standard Deviation
General Features		
Age of Onset (Yrs)	45.33	6.6
Disease Duration(Yrs)	4.23	3.01
Heart Rate (b/m)	86.36	10.61
Pulse Pressure (mm/Hg)	33.40	9.17
Systolic BP (mm/Hg)	110.20	14.35
Diastolic BP (mm/Hg)	70.6	15.06
Hemoglobin (g/dL)	9.96	1.34
Platelet count ($x10^9$/L)	380.58	142.63
Total Leukocytic Count ($x10^3$/µL)	7.64	6.06
ESR (mm/hr)	57.95	27.29
CDAI score	26.43	10.36
HAQ	1.64	0.41
RADAI-5	4.53	0.93

Table (2): H19 SNP (rs2251375) Genotypes and Alleles in Relation to RA Features

Variables	H19 (rs2251375) Genotype			p-value	Alleles		p-value
	CC (n=31)	CA (n=55)	AA (n=14)		C (n=117)	A (n=83)	
General Features							
Age of Onset (Yrs)	46.13	44.75	41.50	0.09	45.66	41.50	0.55
Disease Duration(Yrs)	2.23	2.51	3.75	0.98	4.22	4.26	0.82
Heart Rate (b/m)	79.36	90.50	88.06	**0.04**	90.50	85.00	**0.012**
Pulse Pressure	33.63	35.00	32.81	0.72	35.00	33.26	0.92
Systolic BP (mm/Hg)	104.54	115.00	112.81	0.30	115.00	109.78	0.14
Diastolic BP (mm/Hg)	70.90	77.50	76.25	0.67	77.50	74.43	0.39
Hemoglobin (g/dL)	9.74	10.07	9.55	0.530	9.99	9.50	0.553
Platelet count (10^9/L)	332.18	405.45	296.00	0.644	387.93	296.00	0.18
Total Leukocytic Count ($x10^3$/µL)	6.87	7.98	6.82	0.15	8.01	6.82	0.71
ESR (mm/hr)	54.23	62.98	44.60	**0.04**	58.88	56.79	0.59
CDAI score	23.95	31.75	26.62	0.43	27.19	23.95	0.37
HAQ	1.47	1.69	1.61	0.32	1.69	1.47	0.13
RADAI-5	4.39	5.20	4.52	0.41	4.38	4.58	0.55

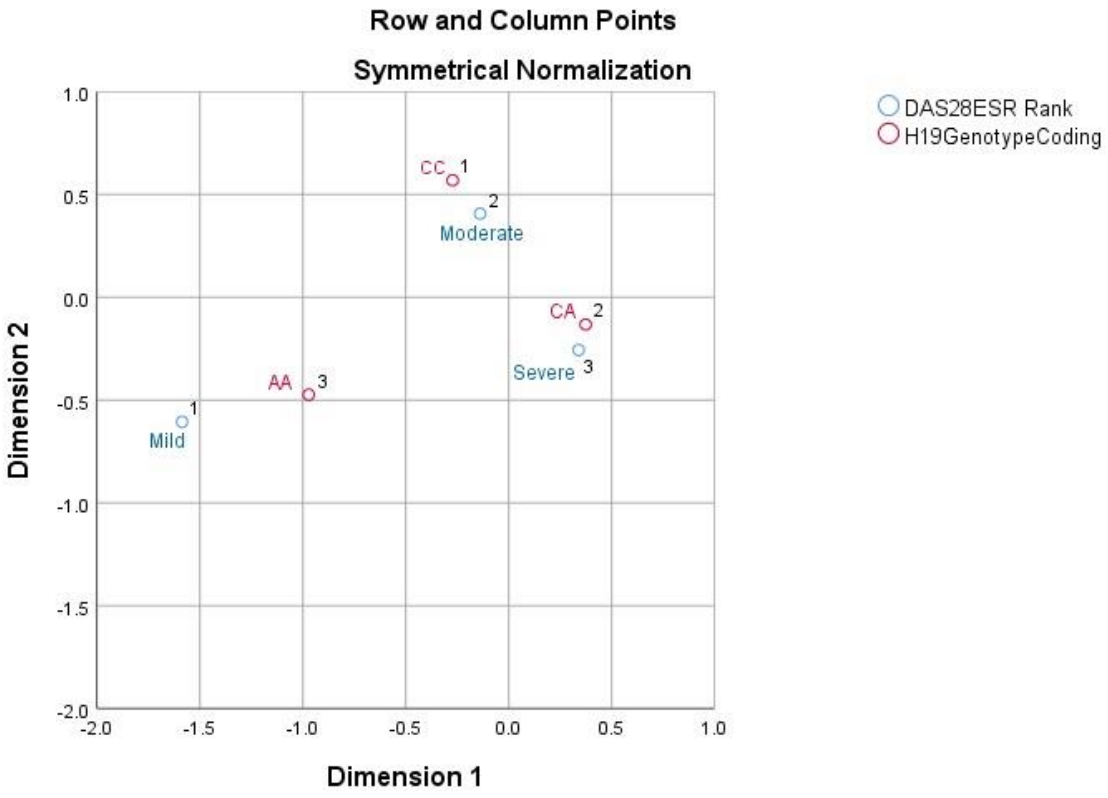

Figure (3): Correspondence Analysis showing distribution of DAS28ESR Ranks according to H19 SNP (rs2251375) Genotypes

Table (3): Regression Analysis using (rs2251375) Heterozygous CA as Inflammation Predictor

Independent Variable	Dependent Variables	R	Unstandardized B	Standardized Coefficients Beta	*p*-value[a]
(rs2251375) Heterozygous CA Genotype	ESR	0.22	12.276	0.222	**0.026**
	Heart Rate	0.33	6.67	0.335	**0.017**

Discussion:

Rheumatoid Arthritis has been a constant field of research due to its complicated nature and elusive etiology. As a systemic autoimmune chronic inflammatory disease that usually leaves an immense impact on global health of the subject, researchers always looked into its genetic nature and possible epigenetic modifications. LncRNAs proved to be an important aspect to be researched as a probable etiological factor and a possible therapeutic target (16).

LncRNA H19 gene expression had been found to be upregulated in RA patients. Its role has been long established in different tissues like synovial, vascular and cardiac tissues (17,18). However, its genetic variants have not been studied in relation to clinical features like heart rate, blood pressure and CDAI before.

In our study, we had genotyped 100 RA patients for H19 SNP (rs2251375) in search for an association with clinical features in the studied group.

Cardiovascular complications in RA patient are celebrated as an intricate multifaceted process. Chronic increased heart rate, inflammation and possible dyslipidemia are some of these factors (11). Our results has shown a significant statistical positive correlation between genotype CA and increased heart rate, which is a finding consistent with H19 increased expression in cardiac muscles postnatally and established H19 role in remodeling process post-cardiovascular events like Coronary Artery Disease (CAD) and Myocardial Infarction (MI) (10).

Taking into consideration how these process are primarily inflammatory in nature, H19 has been labeled as a proatherogenic factor with a significant role in plaque development (10). Increased resting heart rate in itself was studied in relation to inflammatory markers and found to be associated with increased high sensitive CRP, IL-6 and fibrinogen (19). The autonomic nervous system is the golden link between regulating heart rate at rest, immune system regulation and the inflammatory response via tumor necrosis factor (TNF). This cytokine in turn is known to precipitate vascular damage through capillary vessels leakage and enhancement of microvascular thrombosis (19). Resting heart rate had also been found to be an independent risk factor for increased cholesterol levels and higher diastolic blood pressure in healthy obese patients (20).

Additionally, such a finding supports our claim that H19 can easily be considered an inflammatory marker in different diseases. Increased heart rate can either be directly related to inflammation or related to presence of the C allele in studied patients. H19 already had been established as a promoter of vascular inflammation in RA patients (18). However, our study is the first to show an association between increased heart rate in RA patients with the genotype CA and the C allele.

ESR levels were significantly higher in patients with the heterozygous CA genotype with a statistically significant association. Same results with calculated DAS28-ESR score. Our studied SNP was found to be linked with insulin growth factor 2 (IGF2). Additionally, it was also linked to its DNA methylation of the unique H19/IGF2 region (21). Our results that CA genotype is associated with higher ESR levels have found an echo in this study as the researchers found an association between (rs2251375) (A) allele and hypermethylation of H19/IGF2 region which consequently led to an increased expression of IGF2. IGF2 directly contributes to the proliferation RA fibroblast-like synoviocytes (FLS) and is connected with the inflammatory synovium of RA (22)**.**

Chen *et al.* (2021) studied H19 gene expression in yet another celebrated autoimmune inflammatory disease, systemic lupus erythematosus, and found H19 gene is directly associated with SLE disease activity index (SLE-DAI). However, when calculating CDAI in our study, we found no association between the score and RA patients' genotypes. The (A) allele proved to be a direct reason in increased IGF2 expression in a study by **Kondo *et al.* (2022)**. This even leads to a downstream activation of the pro-inflammatory Akt-NF-κB pathway. This pathway significantly increases the expression of inflammatory cytokines' genes such as IL-1β, IL-6 and TNF-α.

On the other hand, other researchers found a non-statistically significant association between this SNP and a number of inflammatory conditions like osteoarthritis, atherosclerotic coronary artery disease and ischemic stroke (25–28)**.**

Ultimately, our findings show an undeniable link between H19 SNP (rs2251375) CA genotype and increased resting heart rate and ESR levels in RA patients which can be a devastating combination leading to impending cardiovascular complication in these patients.

Conclusion:

There is a significant association between H19 SNP (rs2251375) and increased resting heart rate in RA patients and ESR.

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