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The ANRIL rs4977574 Variant is Associated with Rheumatoid Arthritis Susceptibility

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Abstarct

Background: As the most common inflammatory arthritis worldwide, rheumatoid arthritis (RA) has been shown in numerous studies to be significantly influenced by long non-coding RNAs (lncRNAs), particularly in terms of disease onset and progression. This research explores the possible association between *ANRIL* (rs4977574) polymorphisms and susceptibility to RA.

Methods: This study comprised 150 confirmed cases of RA alongside 150 matched healthy controls. Genotyping of DNA was carried out using the tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR).

Results: Based on multivariate logistic regression, carriers of the AG genotype had a 7.13-fold higher risk of developing RA compared to those with the AA genotype. Conversely, the GG genotype was associated with a substantial 0.18-fold reduction in RA risk compared to AA. Additionally, the *ANRIL* rs4977574 variant was shown to increase RA risk across recessive (OR = 1.52, 95% CI = 1.06-2.19, P = 0.022), dominant (OR = 2.49, 95% CI = 1.50-4.13, P = 0.0003), and over-dominant (OR = 8.19, 95% CI = 4.086-16.4, P < 0.0000001) genetic models.

Conclusion: This study established a connection between *ANRIL* genetic variation and an elevated risk of RA within the Iranian population.

Keywords: Rheumatoid arthritis; ANRIL Long non-coding RNA; Tetra-primer ARMS-PCR; Single Nucleotide Polymorphism

1. Introduction

Rheumatoid arthritis (RA) represents an autoimmune condition marked by persistent inflammation affecting the synovial membrane that lines the joints [1]. Genetic factors play a significant role in RA, contributing to approximately 60% of its heritability [2]. Long non-coding RNAs (lncRNAs) make up a considerable portion of the non coding regions of the genome. These molecules are key modulators of inflammatory pathways and play an important role in the onset and progression of autoimmune diseases [3-5]. Among these, lncRNA ANRIL has been linked to inflammation and disease activity in various disorders associated with immunity and inflammation [6].

Emerging evidence has implicated ANRIL in a wide spectrum of inflammatory diseases beyond RA, including systemic lupus erythematosus, inflammatory bowel disease, psoriasis, and type 1 diabetes, highlighting its broad immunoregulatory role [7-11]. The 9p21.3 genomic region, which harbors numerous polymorphisms associated with various diseases, gives rise to the long non coding RNA ANRIL [12]. Zhang et al. analyzed the allele and genotype frequency distribution of ANRIL single-nucleotide polymorphisms (SNPs) (rs1412830, rs944796, rs61271866, rs2518723, and rs3217992) in the Chinese population and found no direct correlation with RA susceptibility. Nevertheless, their results demonstrated a notable link between the frequency of the ANRIL rs944796 G, rs2518723 T, and rs3217992 T alleles and the presence of anti CCP antibodies in RA

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patients, indicating that genetic variants of ANRIL might be involved in the pathogenesis of RA. Furthermore, their results showed that ANRIL expression in peripheral blood mononuclear cells (PBMCs) was significantly lower in RA patients compared to healthy individuals [13]. Falahi et al. investigated ANRIL gene polymorphisms rs1333048 and rs10757278 in the Kurdish population of Iran and found no association with susceptibility to allergic rhinitis, a prevalent inflammatory disorder [14]. In a similar study, Rezazadeh and colleagues investigated the ANRIL rs4977574 polymorphism in Iranian multiple sclerosis patients and found no significant differences in either allele or genotype frequencies when comparing affected individuals to healthy controls [15]. Furthermore, recent functional evidence has shown that ANRIL functions as a competing endogenous RNA (ceRNA) for microRNAs, consequently regulating the expression of inflammatory mediators such as IL 6 and TNF α [16]. Polymorphisms in the ANRIL locus have also been shown to affect its splicing variants and expression levels in PBMCs [17, 18]. However, most of these studies have been conducted in cardiovascular or metabolic diseases, and data on ANRIL genetic variants in RA remain scarce and inconsistent.

Investigating lncRNA variants associated with increased RA susceptibility and disease severity may open new promising pathways for improving diagnostic accuracy and clinical treatment. Such genetic insights may empower clinicians to identify individuals at elevated risk for RA and to tailor therapeutic interventions that optimize drug efficacy and improve patient outcomes. Accordingly, this study examined the rs4977574 SNP of the ANRIL gene, contrasting its occurrence in RA patients versus healthy controls to determine its potential role as a risk factor for rheumatoid arthritis among Iranians. As far as we are aware, this study is the first to directly evaluate the association between the ANRIL rs4977574 variant and susceptibility to rheumatoid arthritis.

Methods

study subject

This study included 150 patients with RA and 150 healthy controls, with ages ranging from 25 to 81 years. Blood samples were obtained from all participants at the Rheumatology Center of Shiraz University of Medical Sciences. The control group (n=150) was selected such that none had a prior history of cancer or autoimmune diseases. RA diagnosis in the patient group was established according to the 2010 classification criteria jointly proposed by the American College of Rheumatology and the European League Against Rheumatism. To enhance comparability, frequency matching was conducted between the RA and control groups, considering age (±5 years) and gender. All participants, both cases and controls, were recruited from a single geographic region (Fars province, southwestern Iran) and were frequency-matched for age and sex. Although this approach does not eliminate the possibility of population stratification, the homogeneous regional background reduces its probability. Ethical approval for the study was obtained from the Ethics Committee of the Islamic Azad University, Marvdasht Branch, Iran (approval code: IR.IAU.M.REC.1402.062). Written informed consent was secured from every participant before enrollment.

SNP genotyping

This study involved the extraction of DNA from whole blood samples provided by participants. DNA extraction was performed using a Favorgen kit (Taiwan). Primers were specifically designed for tetra-primer ARMS-PCR, a method known for its precision and reliability in SNPs. The genotyping of ANRIL polymorphism rs4977574 was carried out using this technique. To confirm the accuracy of genotyping results, PCR products were analyzed via electrophoresis on a 2% agarose gel. Quality control measures ensured no errors, and approximately 20% of the samples were randomly sequenced for validation.

The following primers were used for the detection of rs4977574 polymorphism:

FO: 5'ACCATGCTTTCTGAAACAACACG3'

RO: 5'TGCCCTGAACACGGTAAGC3'

FI: 5'CAGGGTACATCAAATGCATTCTATATCG3'

RI: 5'AGAGTGACTGGAACATCCGGT3'

The size of products included outer primers (485bp), G allele (374bp), and A allele (159bp).

Statistical analysis

To assess the relationship between SNP and RA risk, an unconditional logistic regression analysis was conducted. A pre study power calculation, assuming a minor allele frequency of 0.18 (derived from the control group) and an odds ratio of 2.0 under a dominant model, indicated that 150 cases and 150 controls provide 80% power at $\alpha = 0.05$. As sensitivity analyses, we performed multivariable logistic regression adjusting for age and sex, as well as Firth's penalised logistic regression to reduce potential small-sample bias. Statistical significance was defined as a p-value below 0.05, and all data analyses were carried out using SPSS (version 19.0).

Results

Demographic characteristics

This study included 150 individuals diagnosed with RA, comprising 25 men and 125 women, along with a control group of 150 healthy individuals matched by gender (25 men and 125 women). The mean age did not differ significantly between the RA patient group and the healthy control group (50.91±11.59 years for RA patients versus 49.95±11.34 years for controls, p=0.63). Table 1 summarizes the clinicopathological features of RA patients stratified by sex and age. Notably, RF, CRP, and anti-CCP levels did not differ meaningfully between the sexes.

Table 1. Sex- and age-based distribution of clinicopathological data among RA patients

Characteristics	Sex and Age	Mean ± SD	*P-Value
Rf, mean ± SD (years)	Female	90.11±8.23	0.192
	Male	65.47±5.42	
CRP, mean ± SD (years)	Female	25.58±4.89	0.173
	Male	16.34±3.87	
Anti-CCP, mean ± SD (years)	Female	49.08±8.03	0.164
	Male	35.21±3.75	
Duration, mean ± SD (years)	Female	8.63±1.84	0.532
	Male	9.12±2.51	
Age of disease onset, mean ± SD (years)	Female	42.26±4.37	0.514
	Male	44.23±4.85	

*Independent Samples t- Test

ANRIL rs4977574 Variant and the Risk of RA

Genotypic analysis revealed that among RA patients, the AA, AG, and GG genotypes occurred with frequencies of 88 (58.66%), 59 (39.33%), and 3 (2.00%), respectively. In contrast, the control group exhibited frequencies of 117 (78.00%), 11 (7.33%), and 22 (14.66%). Table 2 outlines the association between the rs4977574 polymorphism genotypes and RA susceptibility within the Iranian population. Multivariate logistic regression analysis indicated that individuals possessing the AG genotype of the rs4977574 polymorphism exhibited a significantly increased susceptibility to RA, with an odds ratio (OR) of 7.13 compared to those with the AA genotype (OR = 7.13, 95% CI = 3.53–14.37, $p < 0.0000001$). Conversely, the GG genotype was associated with a notable 0.18-fold reduction in RA risk compared to AA ($p = 0.002$). Further analysis based on the recessive genetic model revealed that individuals carrying the GG genotype exhibited a significantly elevated risk of developing RA (OR = 1.52, 95% CI = 1.06–2.19, $p = 0.022$). In the over-dominant model, individuals with the AG genotype had an 8.19-fold higher risk of RA (OR = 8.19, 95% CI = 4.08–16.4, $p < 0.0000001$). Additionally, the presence of GG+AG genotypes was associated with a 2.49-fold increased risk of RA under the dominant model (OR = 2.49, 95% CI = 1.50–4.13, $p = 0.0003$). However, no significant difference was observed in allele frequencies of the ANRIL rs4977574 polymorphism between RA patients and controls ($p > 0.05$).

A post hoc power analysis using the observed AG genotype frequency in controls (7.3%) and the OR of 8.19 (from the over-dominant model) yielded >99% power, confirming the study's adequate sample size to detect the observed effect.

Discussion

lncRNA ANRIL has garnered significant attention in autoimmune diseases due to its role in regulating immune and inflammatory responses [15]. The regulation of its expression occurs via the STAT1 signaling cascade, which plays a role in immune modulation through the induction of the proinflammatory cytokine interferon-gamma (INF-γ) [19]. ANRIL is involved in RNA interference, gene silencing, and epigenetic mechanisms, influencing the expression of various genes. This lncRNA has been linked to multiple immune-related conditions, including RA and coronary artery disease (CAD). For instance, in patients with inflammatory bowel disease, the expression level of ANRIL correlated with CRP concentrations [6]. Similarly, in psoriasis vulgaris, specific ANRIL SNPs were associated with disease susceptibility and severity [12]. Another study of ANRIL polymorphisms across multiple inflammatory disorders confirmed that the 9p21.3 locus is a shared risk region, although effect sizes vary considerably across ethnic groups [20]. While genome-wide association studies have explored the genetic characteristics of ANRIL in inflammatory diseases, research specifically investigating its involvement in RA pathogenesis remains limited [12].

A recent investigation revealed that activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway in stimulated B lymphocytes results in elevated ANRIL expression. Furthermore, upon treatment with TNF-α, ANRIL knockdown was shown to upregulate the expression of IL-6 and IL-8 [21]. Considering the roles of the STAT1, NF-κB, and TNF-α signaling pathways in immune responses and the pathogenesis of RA [22], along with their reported interactions with ANRIL, the present study aimed to investigate the ANRIL rs4977574 variant in both RA patients and healthy controls to assess its potential association with susceptibility to RA in the Iranian population.

Table 2. Evaluation of rs4977574 Genotypes in Patients with Rheumatoid Arthritis versus Healthy Individuals under Different Inheritance Models

SNP	Genotype	Inheritance model	Patient group (%)	Control group (%)	OR (95% CI)	P-value
rs4977574	AA	Codominant	88 (58.66)	117 (78.00)	1	
	AG		59 (39.33)	11 (7.33)	7.13 (3.53-14.37)	<0.0000001
	GG		3 (2.00)	22 (14.66)	0.18 (0.05-0.62)	0.002
	AA+AG	Recessive	147 (98.00)	128 (85.33)	1	
	GG		3 (2.00)	22 (14.66)	1.52 (1.06-2.19)	0.022
	AA+GG	Over-dominant	91 (60.66)	139 (92.66)	1	
	AG		59 (39.33)	11 (7.33)	8.19 (4.086-16.4)	<0.0000001
	AA	Dominant	88 (58.66)	117 (78.00)	1	
	GG+AG		62 (41.33)	33 (22.00)	2.49 (1.50- 4.13)	0.0003
	A	Allele	235 (78.33)	245 (81.66)	1	
	G		65 (21.66)	55 (18.33)	1.23 (0.82- 1.84)	0.30

Although the present study establishes a genetic association between the ANRIL rs4977574 variant and RA risk, functional validation is required to elucidate the underlying biological mechanism. The rs4977574 variant within an intronic region of the 9p21.3 locus. Despite being a non-protein-coding variant, it may directly affect ANRIL gene expression[23]. Previous studies have established a link between ANRIL and NF-κB signaling pathways, indicating that ANRIL modulates the expression of IL6 and IL8 through its interaction with a transcription factor [24]. Therefore, it is plausible that rs4977574 modulates ANRIL expression levels or isoform splicing, thereby influencing inflammatory responses. However, direct experimental evidence is needed to confirm this hypothesis.

Several limitations warrant acknowledgment. First, the study's patient samples were obtained from a single referral center in Shiraz, located in Fars province (southwest Iran). Although the population of this region is predominantly Persian, Iran is characterized by high genetic diversity, including distinct ethnic groups. Therefore, our findings may not be generalizable to the entire Iranian population or to other ethnicities. We attempted to control for population stratification by matching cases and controls for age, sex, and geographic origin (all from Fars province). Nevertheless, larger multi-center studies that include diverse ethnic groups from across Iran are required to determine whether the observed association holds true at the national level. Until such replication studies are performed, our results should be considered specific to the population of southwest Iran. Second, the relatively limited sample size, along with the potential impact of environmental factors, may have influenced the findings. Moreover, the large effect sizes observed in this study, particularly the OR of 8.19 in the over dominant model, may be overestimated due to the modest sample size. Consequently, these findings should be considered hypothesis generating, and independent replication in larger, multi centre cohorts is essential before definitive conclusions can be drawn. Lastly, the present study did not evaluate the effect of the ANRIL variant on its expression levels or functional activity. Therefore, we strongly recommend further investigations with larger sample sizes to determine whether ANRIL SNPs, particularly rs4977574, influence gene expression and contribute to RA development. Also, functional studies are essential to confirm the biological plausibility of the observed genetic association.

This population-based case-control study represents, to our knowledge, the first report proposing a potential involvement of the ANRIL rs4977574 polymorphism in RA susceptibility. Our findings demonstrate that the AG genotype is linked to an increased risk of RA, while the GG genotype appears to confer protection against disease development. However, these findings are limited to the population of southwest Iran and require validation in other ethnic groups and larger cohorts. Expanding such studies could offer valuable insights and further enhance our understanding of this association.

Author contributions

A.K. carried out the experiments. M.J.M. conceived and designed the study, analyzed the data, wrote the manuscript, and supervised the research. All authors have read and approved the final manuscript.

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Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Data Availability Statement

The datasets are available from the corresponding author upon reasonable request.

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