

# Association of RIPK1 and RIPK2 Gene Polymorphisms with Rheumatoid Arthritis in a Chinese Han Population

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**Objects:** Rheumatoid arthritis (RA) is a systemic autoimmune disease with an obscure pathogenesis. This study aims to identify the susceptibility conferred by specific single nucleotide polymorphisms (SNPs), namely rs17548629 within the RIPK1 gene and rs10094579 within the RIPK2 gene, in RA. Additionally, it investigates the associations between inflammatory markers and biochemical parameters at various stages of the disease.

**Methods:** We analyzed 394 patients with RA and 258 normal controls (NCs), examining SNPs within the RIPK1 (rs17548629) and RIPK2 (rs10094579) genes using polymerase chain reaction (PCR) and sequencing techniques. Profiles of RA patients were evaluated for inflammatory markers, including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), as well as biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, glucose, uric acid, and creatinine. Additionally, disease-specific indicators included cyclic citrullinated peptide (CCP), rheumatoid factor (RF), antinuclear antibodies (ANA), and anti-keratin antibodies. The Disease Activity Score 28 (DAS28), based on ESR, was used to categorize RA patients into groups of high, moderate, or low disease activity.

**Results:** We found a significant association between the RIPK1 rs17548629 genotype and RA in the additive model ( $p < 0.001$ ; OR = 3.23), over-dominant model ( $p < 0.001$ ; OR = 0.27), and dominant model ( $p < 0.001$ ; OR = 3.94). The frequency of the C allele at rs17548629 was significantly higher in NCs than in RA patients ( $p < 0.001$ ; OR = 0.322). When compared with normal controls, the RIPK1 rs17548629 genotype demonstrated significant associations with both anti-CCP-positive RA patients ( $p < 0.001$ ) and anti-CCP-negative RA patients ( $p < 0.001$ ). Similarly, this genotype was associated with RF-positive RA patients ( $p < 0.001$ ). Furthermore, the RIPK2 rs10094579 genotype was significantly associated with CRP levels in RA patients with low disease activity in the over-dominant model ( $p = 0.029$ ; OR = 0.065, adjusted for age and sex).

**Conclusion:** The presence of the RIPK1 rs17548629 genotype is associated with RA under additive, co-dominant, and dominant models. The T allele mutation at rs17548629 increases the risk of RA in the Chinese population. The RIPK1 rs17548629 genotype was identified as being associated with RF-positive RA patients, whereas no significant association was observed in RF-negative individuals. These findings suggest that this SNP may modulate the risk of RA in an RF-dependent manner. Furthermore, the RIPK2 rs10094579 genotype correlates with CRP levels in RA patients exhibiting low disease activity. This association underscores the necessity for caution when reducing the dosage of therapy in RA patients with low disease activity who carry the CA genotype at RIPK2 rs10094579. Additional research is warranted to explore other genotypes that may influence RA susceptibility and to refine potential treatment strategies.

**keywords:** receptor-interacting protein kinase, rheumatoid arthritis, polymorphism, single nucleotide, biochemical parameters

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by synovial inflammation, articular cartilage degradation, and bone erosion.<sup>1</sup> RA significantly affects patients' quality of life, and the pathogenesis of RA remains

poorly understood. Cytokines play a crucial role in immune responses, mediating cellular differentiation and inflammation in affected joints. Current research highlights the essential involvement of nuclear factor- $\kappa$ B (NF- $\kappa$ B),<sup>2</sup> nucleotide-binding oligomerization domain (NOD),<sup>3</sup> and tumor necrosis factor (TNF) in the pathogenesis of RA. Although the etiology of RA is still elusive, genetic predispositions have been implicated in its development, particularly when combined with environmental triggers.<sup>4</sup>

Receptor-interacting protein kinase (RIPK) is a family of seven members sharing homologous serine-threonine kinase domains.<sup>5</sup> Among these, RIPK1, RIPK2, and RIPK3 have emerged as master regulators of inflammatory signaling.<sup>6</sup> The blockade of RIPK1 presents a promising strategy for mitigating inflammation.<sup>7,8</sup> Studies indicate that RIPK1 inhibition ameliorates the inflammatory response and suppresses osteoclastogenesis in collagen-induced arthritis (CIA) by inhibiting necroptosis.<sup>9</sup> Small-molecule RIPK1 inhibitors have progressed to phase 2a clinical studies for conditions like psoriasis, RA, and ulcerative colitis.<sup>10</sup> RIPK2, initially identified in 1998, specifically interacts with tumor necrosis factor TNF and mediates NF- $\kappa$ B activation and cell death.<sup>11</sup> RIPK2 is a critical mediator of inflammatory responses via intracellular peptidoglycan sensors known as NOD proteins.<sup>12</sup> Excessive NOD signaling is implicated in various diseases, including inflammatory bowel disease, sarcoidosis, and RA.<sup>13,14</sup> Furthermore, evidence indicates that RIPK2 deficiency impairs T cell proliferation and differentiation into the T helper 1 (TH1) subset<sup>15</sup> and may also induce dysfunction in cytotoxic T lymphocytes.<sup>16</sup> RIPK3 can form a complex with tumor necrosis factor receptor 1 (TNFR1), thereby inducing necroptosis through its interaction with RIPK1.<sup>17</sup>

In our previous study, we observed elevated plasma levels of RIPK1 in patients with RA compared to healthy controls. Additionally, the administration of a RIPK1 inhibitor significantly mitigated joint swelling, bone destruction, tissue damage, and plasma levels of inflammatory cytokines in rats with CIA.<sup>18</sup> Despite the proven clinical efficacy of RIPK inhibition therapy, the role of RIPK1 single nucleotide polymorphisms (SNPs) in RA remains unclear. SNPs, which represent variations at specific genomic sites, are the most prevalent type of DNA sequence variation and can affect gene expression, protein functionality, and disease susceptibility.<sup>19</sup> Genetic variants of RIPK1 might alter its substrate binding, transcription activation, and apoptotic induction capabilities.<sup>20</sup> RIPK1-deficient mice demonstrate multiple tissue defects, resulting in systemic inflammation and perinatal mortality within the first three days of life.<sup>21</sup> Previous research identified four individuals from three unrelated consanguineous families with homozygous loss-of-function mutations in RIPK1, presenting with lymphopenia, recurrent infections (viral, bacterial, fungal), early-onset inflammatory bowel disease (both upper and lower GI tracts), and arthritis.<sup>22</sup> Genetic factors are paramount in RA development,<sup>23</sup> underscoring the importance of identifying genetic susceptibility markers for predicting RA risk. This study aims to investigate the associations between RIPK1 (rs17548629) and RIPK2 (rs10094579) gene polymorphisms in RA patients, exploring their correlation with inflammatory markers and blood biochemical parameters across various RA disease stages.

## Methods

### Study Subjects

Between April 2016 and June 2022, we enrolled 394 RA patients at the China-Japan Friendship Hospital in Beijing. To qualify for inclusion, patients were required to meet the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 criteria.<sup>16</sup> We excluded patients with other autoimmune diseases or those under the age of 18. Additionally, patients whose Disease Activity Score 28 (DAS28) could not be calculated due to missing information were also excluded. The control group consisted of 258 individuals of the same Chinese ethnic origin, recruited from the physical examination center at the hospital. None of the control individuals or their family members had a history of autoimmune diseases. All study protocols involving human subjects received approval from the ethics committee of the China-Japan Friendship Hospital (ethics ID: 2015–16 and 2014–58), ensuring compliance with the Declaration of Helsinki.

## DNA Extraction

We collected 1 mL of peripheral blood from each patient with RA and NCs using the standard venipuncture technique for genetic analyses. Following centrifugation, white blood cells were isolated and stored at  $-80^{\circ}\text{C}$  for subsequent experiments. DNA was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). We quantified DNA samples and assessed their purity using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), ensuring adequacy for genotyping analyses. Genomic DNA size was verified by 1% agarose gel electrophoresis.

## SNP Genotyping

Polymerase chain reaction (PCR) was utilized to amplify gene polymorphisms, employing specifically designed forward and reverse primers via Primer 5 software. The primer sequences were as follows: RIPK1: forward 5'-AAACATCAGAATTTCAGG-3', reverse 5'-CTTTTTCACCTCTGGCACACA-3'; RIPK2: forward 5'-TTGCTAGTGTCTCTGAATAC-3', reverse 5'-GATGATTCACAAAGACCGTT-3'. The PCR mixture contained 15  $\mu\text{L}$  of master mix, 1  $\mu\text{L}$  of DNA template, 12  $\mu\text{L}$  of ddH<sub>2</sub>O, and 1  $\mu\text{L}$  of each primer, totaling 30  $\mu\text{L}$ . Thermal cycling conditions were initiated with a denaturation step at  $94^{\circ}\text{C}$  for 5 minutes, followed by 20 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $65^{\circ}\text{C}$  for 30 seconds, and  $72^{\circ}\text{C}$  for 30 seconds. This was succeeded by 15 cycles at  $94^{\circ}\text{C}$  for 30 seconds,  $55^{\circ}\text{C}$  for 30 seconds, and  $72^{\circ}\text{C}$  for 30 seconds, concluding with a final extension at  $72^{\circ}\text{C}$  for 7 minutes. Gene polymorphisms were identified through sequencing, and SNP assay results were analyzed using Chromas version 2.23 (Technelysium Pty Ltd., Brisbane, Australia) for absolute quantification. All selected SNPs from the NCBI Database of Short Genetic Variations (dbSNP) exhibited minor allele frequencies (MAF) above 0.02. Statistical power was calculated with Quanto Version 1.2.4, achieving a value exceeding 0.80.

## Routine Blood Examinations

Biochemical markers were assayed using the Beckman AU5800 automatic clinical chemistry analyzer (Beckman Coulter, Inc., USA). The enzymatic colorimetric method facilitated the detection of alanine transaminase (ALT), aspartate transaminase (AST), glucose (GLU), uric acid (UA), and creatinine (CR). Furthermore, C-reactive protein (CRP) levels, with a reference of less than 0.8 mg/dL, and rheumatoid factor (RF) levels, with a reference of less than 20 IU/mL, were quantified using the immunoturbidimetric immunoassay method. The erythrocyte sedimentation rate (ESR) was determined via Westergren's method utilizing the VACUETTE RSR100/II Automatic Sed-rate Analyzer (Greiner Bio-One GmbH, Austria). Levels of cyclic citrullinated peptide (CCP) were measured using an enzyme-linked immunosorbent assay (Euro Diagnostica, Spain).

## Statistical Analysis

We conducted the Shapiro–Wilk test to evaluate the normality of our data set. Quantitative clinical data, including patient age, DAS28, and ALT, were characterized using median and interquartile ranges (IQRs). These data were subsequently analyzed using tests appropriate for non-normal distributions. For categorical clinical data, such as SNP genotyping, CCP antibodies, and RF, we employed counts and frequencies followed by Fisher's exact test or chi-square test, as deemed appropriate. We assessed ethnic and gender disparities in allelic genotype frequencies among RA patients and non-affected controls (NCs) using Hardy-Weinberg equilibrium. Logistic regression analyses exploring the association between gene polymorphisms and RA risk were performed in additive, recessive, dominant, and over-dominant models, adjusting for age and sex. For instance, in the additive model for RIPK1 rs17548629, where "T" is the minor allele, genotypes CC, CT, and TT were assigned values of 0, 1, and 2, respectively. In the dominant model, genotypes CT and TT were coded as 1, while CC remained 0. Conversely, in the recessive model, genotypes CC and CT were coded as 0, and TT as 1. In the over-dominant model, genotypes CC and TT received a code of 0, and CT was coded as 1. To assess RA disease activity, we used the DAS28, calculated as follows:  $0.56 * \sqrt{28 \text{ tender joint count}} + 0.28 * \sqrt{28 \text{ swollen joint count}} + 0.70 * \ln(\text{ESR}) + 0.014 * (\text{visual analogue scale, VAS})$ . On this scale, 0 represents the best

possible condition and 100 the worst. Based on DAS28 scores, RA patients were categorized into high ( $\text{DAS28} > 5.1$ ), moderate ( $3.2 < \text{DAS28} \leq 5.1$ ), and low ( $\text{DAS28} \leq 3.2$ ) disease activity groups.<sup>24</sup>

We conducted all statistical analyses using the Statistical Package for the Social Sciences (SPSS) version 27 (IBM Corp., Armonk, NY, USA). Additionally, we employed QUANTO software to assess the power of the study and determine the necessary sample size (available at <http://hydra.usc.edu/gxe/>). We considered  $p$ -values less than 0.05 as statistically significant.

## Results

### Clinical Parameters of the Study Population

Our study encompassed 258 NCs, with 194 (75.19%) females and 64 (24.81%) males. Among the 394 RA patients, comprising 315 (79.95%) females and 79 (20.05%) males. The average age was  $56.04 \pm 9.91$  years for NCs and  $56.28 \pm 14.31$  years for RA patients. Statistical analysis revealed no significant age differences between the RA patients and NCs ( $p = 0.188$ ) or in gender distribution ( $p = 0.151$ ), as detailed in Table 1, which describes the demographic characteristics of the RA cohort.

### RIPK1 rs17548629 Genotype is Associated with RA

Each SNP in both the NC and RA groups was in Hardy-Weinberg equilibrium ( $p > 0.05$ , as shown in Table 2), confirming that our study sample adequately represents the general population. We identified a significant association between the RIPK1 rs17548629 genotype and RA using the chi-square test ( $p < 0.001$ , detailed in Table 2). Additionally, the frequency of the C allele at rs17548629 was significantly higher in the NC group than in the RA group ( $p < 0.001$ ; OR = 0.322) (detailed in Table 2). Importantly, the presence of the T allele at the RIPK1 rs17548629 locus was significantly associated with an increased risk of RA under various genetic models, including additive, dominant, and

**Table 1** Clinical Parameters of the Study Population

	RA patient			NC	p-value*
	High disease activity	Moderate disease activity	Low disease activity		
Age(years)	60.0(51.0–68.5)	56.0(45.3–64)	49.0(37.0–62.0)	55.0(49.0–63.0)	0.188
Sex(female/male)	152/45	133/27	30/7	194/64	0.151
ALT (IU/L)	16(11–24.25)	16(12–24)	18(13–27.75)	19(14–26)	<0.001
AST(IU/L)	16(14–21.25)	17(14–23)	17(15–23)	21(18–25)	<0.001
UREA (mmol/L)	4.90(3.98–6.03)	4.87(3.85–6.15)	4.85(4.13–5.54)	4.57(3.81–5.40)	0.003
GLU(mmol/L)	5.20(4.68–5.87)	5.03(4.69–5.56)	4.98(4.75–5.35)	5.72(5.36–6.41)	<0.001
UA (umol/L)	258(205–325.75)	264(214–319.75)	216.50(189.25–300)	299(249–344)	<0.001
CR (umol/L)	53.90(47.08–66.43)	56.2(50–66.55)	58.05(50.3–67.85)	60.8(54.50–70)	<0.001
CRP(mg/dL)	3.10(1.09–6.98)	1.23(0.57–2.54)	0.34(0.17–0.84)		
ESR(mm/h)	57.00(31.00–84.50)	26.5(16.00–45.00)	8(5.5–14.5)		
VAS	30.00(25.00–50.00)	20(10–30)	10(10–15)		
Tender joint count	19(13–24)	4(2–8.75)	2(1–2)		
Swollen joint count	11(2–22)	1(0–2)	0(0–1)		
Morning stiffness (minutes)	30(0–60)	0(0–30)	0(0–2.5)		
Duration of disease (years)	5(1–10.5)	5(2–10)	6(2.5–12)		
DAS28	6.33(5.68–7.10)	4.13(3.58–4.72)	2.59(2.14–2.82)		
CCP Positive no.(%)	153(81.38%)	126(85.14%)	28(82.35%)		
RF Positive no.(%)	186(95.88%)	147(94.84%)	33(91.67%)		
ANA Positive no.(%)	109(60.22%)	84(59.57%)	18(62.07%)		
AKA Positive no.(%)	101(59.76%)	69(53.90%)	20(64.52%)		

**Notes:** \*:The  $p$ -value is compared between RA and NC group.

**Abbreviations:** RA, rheumatoid arthritis; NC, normal control; ALT, alanine transaminase; AST, aspartate transaminase; GLU, glucose; UA, uric acid; CR, creatinine; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; VAS, visual analog scale; DAS28, Disease activity score 28; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; ANA, antinuclear antibody; AKA, anti keratin antibody. Quantitative clinical data, such as age, DAS28, and ALT were described using median and interquartile range.

**Table 2** Allele Frequencies and Hardy-Weinberg in RA Patients and NC

	RA	NC	p-value	OR (95% CI)
	(n=394)	(n=258)		
RIPK1				
rs17548629 genotype				
C	591	466		
T	197	50	<0.001	0.322(0.231–0.449)
p-value in Hardy-Weinberg	0.075	0.067		
RIPK2				
rs10094579 genotype				
C	613	407		
A	171	107	0.669	0.942(0.718–1.237)
p-value in Hardy-Weinberg	0.689	0.419		

**Abbreviations:** RA, rheumatoid arthritis; NC, normal control; OR, odds ratio; CI, confidence interval; Chi square was used in Table 2.

**Table 3** Genotype and Allele Frequencies Between RA Patients and NC

	Genotype	RA	NC	p-value	Unadjusted OR (95% CI)	Adjusted p-value <sup>a</sup>	Adjusted OR (95% CI) <sup>a</sup>
		(n=394)	(n=258)				
<b>RIPK1 (rs17548629)</b>	CC	215	213	-	-	-	-
	CT	161	40	<0.001	3.99(2.71–5.98)	<0.001	3.98(2.71–5.98)
	TT	18	5	0.014	3.57(1.39–10.96)	0.017	3.42(1.33–10.53)
	CC vs TT			0.013	3.567(1.301,9.780)	0.018	3.387(1.231,9.319)
	Additive model <sup>b</sup>			<0.001	3.23(2.31–4.62)	<0.001	3.21(2.29–4.60)
	Dominant model						
	CC	215	213	-	-	-	-
	CT+TT	179	45	<0.001	3.94(2.72–5.80)	<0.001	3.92(2.71–5.77)
	Recessive model						
	TT	18	5	-	-	-	-
<b>RIPK2(rs10094579)</b>	CT+CC	376	253	0.08	0.41(0.13–1.05)	0.10	0.43(0.14–1.10)
	Over-Dominant model						
	CT	161	40	-	-	-	-
	CC+TT	233	218	<0.001	0.27(0.18–0.39)	<0.001	0.26(0.18–0.39)
	CC	241	159	-	-	-	-
	CA	131	89	0.864	0.97(0.69–1.36)	0.868	0.97(0.69–1.36)
	AA	20	9	0.356	1.47(0.67–3.46)	0.354	1.47(0.67–3.47)
	CC vs AA			0.356	1.466(0.651,3.302)	0.351	1.472(0.653,3.316)
	Additive model <sup>b</sup>			0.668	1.06(0.81–1.40)	0.664	1.06(0.81–1.40)
<b>RIPK2(rs10094579)</b>	Dominant model						
	CC	241	159	-	-	-	-
	CA+AA	151	98	0.921	1.02(0.74–1.41)	0.916	1.02(0.74–1.41)
	Recessive model						
	AA	20	9	-	-	-	-
	CA+CC	372	248	0.337	0.68(0.29–1.47)	0.336	0.67(0.29–1.46)
	Over-Dominant model						
	CA	131	89	-	-	-	-
	CC+AA	261	168	0.750	1.06(0.76–1.47)	0.753	1.05(0.76–1.47)

**Notes:** Logistic regression analyses was used in Table 3. Dominant model: CT+TT vs CC; Recessive model: CC+CT vs TT; over-Dominant model: CC+TT vs CT; a: Logistic regression with adjustment for age and sex; b: If minor allele is 'T', in the additive model, genotypes CC, CT, and TT were assigned values of 0, 1, and 2, respectively.

**Abbreviations:** RA, rheumatoid arthritis; NC, normal control; OR, odds ratio; CI, confidence interval.

over-dominant, as well as in the comparison of CC versus TT genotypes, with adjustments for age and sex (detailed in Table 3).

Specifically, individuals carrying the T allele (T/T and C/T) of SNP rs17548629 were found to be four times more susceptible to RA compared to those without the T allele in our study (AOR: 3.922, 95% CI: 2.71 to 5.77;  $p < 0.001$ ).

Additionally, individuals with the rs17548629 TT genotype exhibited a threefold higher risk of RA than those with the CC genotype (AOR: 3.387, 95% CI: 1.231 to 9.319;  $p = 0.018$ ). Conversely, the RIPK2 rs10094579 genotype was not associated with RA susceptibility in this cohort, irrespective of adjustments for age and sex.

### Correlation Between SNPs and the Autoantibodies Status in RA Patients

We investigated the associations of the RIPK genotype with autoantibody-positive and autoantibody-negative RA separately. Our findings indicate that, compared to NCs, the RIPK1 rs17548629 genotype is significantly associated with both CCP-positive RA ( $p < 0.001$ , Table 4) and CCP-negative RA ( $p < 0.001$ , Table 4). Similarly, this genotype shows a significant association with RF-positive RA ( $p < 0.001$ , Table 4), but not with RF-negative RA ( $p = 0.452$ , Table 4). However, no significant relationship was found between the RIPK2 rs10094579 genotype and either autoantibody-positive or autoantibody-negative RA.

### Correlation Between SNPs and Inflammation and Biochemical Parameters in Different Disease Activity of RA Patients

We observed that the RIPK1 rs17548629 genotype was associated with CRP levels in RA patients exhibiting low disease activity ( $p = 0.021$ , as presented in Table 5). Furthermore, the RIPK2 rs10094579 genotype demonstrated an association with CRP levels in RA patients with low disease activity ( $p = 0.029$ , displayed in Table 5). Considering the T allele as the minor allele for both RIPK1 rs17548629 and RIPK2 rs10094579, we examined the relationship between these SNPs and CRP levels, employing both dominant and over-dominant models. Our analysis indicated that the RIPK1 rs17548629 genotype association with CRP levels in RA patients exhibiting low disease activity persisted in the dominant model ( $p = 0.069$ , OR: 5.338, 95% CI: 0.880–32.377, adjusted for age and sex) and the over-dominant model ( $p = 0.235$ , OR: 2.701, 95% CI: 0.523 to 13.936, adjusted for age and sex). Likewise, the RIPK2 rs10094579 genotype showed an association with CRP levels in

**Table 4** Associations Between Selected Polymorphisms in the RIPK Genes and Autoantibodies-Positive or Autoantibodies-Negative RA

Group		rs17548629	rs10094579
NC	pp	213	159
	pq	40	89
	qq	5	9
CCP-positive RA	pp	170	189
	pq	120	103
	qq	17	13
	p-value	<0.001	0.889
CCP-negative RA	pp	33	35
	pq	29	22
	qq	1	6
	p-value	<0.001	0.148
RF-positive RA	pp	196	224
	pq	152	122
	qq	18	18
	p-value	<0.001	0.677
RF-negative RA	pp	14	10
	pq	5	9
	qq	0	0
	p-value	0.452	0.513

**Notes:** p-value was calculated through using the Fisher's exact test or chi-square test compared with NC.

**Abbreviations:** RA, rheumatoid arthritis; NC, normal control; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; p- major allele; q- minor allele;



**Table 5** Association of RIPK with Inflammation and Biochemical Parameters in Different Disease Activity of RA Patients

		Low disease activity		Moderate disease activity		High disease activity	
		Rs17548629	Rs10094579	Rs17548629	Rs10094579	Rs17548629	Rs10094579
ALT	pp	17.5(13–24.75)	18(12–28)	16(13–24)	19(13–25.5)	16(11–21.25)	16(11–23.5)
	pq	23(14–28)	18(13–23)	15(10–22.5)	14.5(10–19.75)	15.5(11–26)	16(12–23)
	qq	38(38–38)	20(18–22)	20(18–24)	16.5(11.25–18.75)	15.5(13–18)	15.5(9.75–20)
	p-value	0.17	0.988	0.229	0.065	0.925	0.918
AST	pp	16(15–21.75)	16(15–22)	18(15–23)	18(15–23.5)	16(14–20.25)	16(14–22)
	pq	19(15–23)	18(15–25)	17(13–21.5)	17(13.75–23)	16(13.25–22.75)	17(14–21)
	qq	30(30–30)	18.5(16.25–20.75)	20(16–23)	16(14.75–18.25)	17(15.75–18.5)	14.5(12.25–19.5)
	p-value	0.332	0.963	0.150	0.214	0.756	0.578
UREA	pp	4.75(4.11–5.41)	5.14(4.51–6.54)	4.87(4.12–5.89)	4.86(3.79–6.02)	4.66(4.05–5.87)	4.74(4.02–6.03)
	pq	5.03(3.7–5.36)	4.42(4.12–5.32)	4.92(3.79–6.15)	4.91(3.99–6.00)	5.15(3.89–6.05)	4.99(3.96–5.77)
	qq	5.52(5.52–5.52)	3.98(3.74–4.21)	4.21(3.33–7.99)	4.16(3.56–5.07)	4.51(3.99–5.40)	5.35(4.1–5.88)
	p-value	0.644	0.091	0.868	0.555	0.868	0.777
GLU	pp	4.89(4.74–5.3)	4.99(4.78–5.32)	4.96(4.56–5.52)	4.99(4.72–5.58)	5.22(4.85–5.86)	5.09(4.60–5.92)
	pq	5.12(4.96–5.24)	4.86(4.76–5.32)	5.12(4.85–5.46)	5.14(4.79–5.64)	5.20(4.5–5.85)	5.3(4.85–5.65)
	qq	5.32(5.32–5.32)	3.98(3.74–4.21)	5.26(5–5.64)	4.83(4.62–5.16)	4.77(4.54–5.45)	5.36(4.69–7.68)
	p-value	0.301	0.947	0.196	0.425	0.447	0.555
UA	pp	223(193.5–295.75)	288(195–312)	265.5(225.5–326.25)	264(214.5–318.5)	258(223.25–332.25)	245(206.5–307)
	pq	210(171–301)	211(188–228)	264(214.5–312)	259(212.5–316)	252.5(191.5–322)	274(212–351)
	qq	201(201–201)	195.5(168.25–222.75)	249(201–254)	255.5(231.5–326.25)	258.5(228.25–269.75)	250(197–348.5)
	p-value	0.598	0.218	0.392	0.980	0.668	0.145
CR	pp	58.05(50.3–66.35)	59.8(52.9–68.9)	57.5(50–66.1)	57.3(50.6–65.75)	54.9(48.05–67.35)	53.8(47.25–65.5)
	pq	59.8(52.9–68.9)	51.4(46.7–66.5)	55.7(50.05–67.85)	54.7(49.35–66.68)	52.8(45.88–63.13)	55.9(47.3–67.2)
	qq	50.6(50.6–50.6)	55.75(52.28–59.22)	53.2(50.8–64.7)	69.7(55.83–78.73)	63.5(53.03–82.63)	51.9(46.45–58.78)
	p-value	0.723	0.375	0.951	0.211	0.183	0.847
CRP	pp	0.19(0.14–0.56)	0.56(0.23–1.04)	1.15(0.41–2.36)	1.08(0.65–2.27)	2.74(1.09–7.36)	3.33(1.12–8.16)
	pq	0.8(0.29–0.86)	0.2(0.13–0.28)	1.36(0.78–2.71)	1.49(0.57–2.65)	4.11(1.11–6.98)	2.18(0.9–5.38)
	qq	1.39(1.39–1.39)	0.97(0.97–0.97)	0.98(0.8–2.53)	0.57(0.19–1.07)	1.89(1.15–2.97)	4.83(2.99–6.68)
	p-value	0.021*	0.029*	0.497	0.157	0.326	0.058
ESR	pp	8(5.5–11)	8(7–17)	22(13–43.5)	25(14–43)	53(30–84.75)	60(32.5–83.5)
	pq	7.5(4.75–16.75)	7.5(5–10)	28(19.5–46.5)	30.5(19–48)	63(36.5–83)	44(29.5,80.5)
	qq	12(12–12)	8(7.5–8.5)	37(29–39)	22.5(15.25–39.5)	31.5(26.25–73)	60(52.5–82)
	p-value	0.770	0.759	0.153	0.355	0.308	0.264

**Note:** \*p-values less than 0.05. p- major allele; q- minor allele; p-value was calculated through non-Gaussian tests.

**Abbreviations:** RA, rheumatoid arthritis; NC, normal control; ALT, alanine transaminase; AST, aspartate transaminase; GLU, glucose; UA, uric acid; CR, creatinine; CRP, C reactive protein; ESR, erythrocyte sedimentation rate.

RA patients exhibiting low disease activity in the dominant model ( $p = 0.063$ , OR: 0.141, 95% CI: 0.018–1.113, adjusted for age and sex) and the over-dominant model ( $p = 0.029$ , OR: 0.065, 95% CI: 0.006 to 0.759, adjusted for age and sex). Notably, heterozygous individuals (CA) had a mean CRP level of  $0.32 \pm 0.28$ , while homozygous individuals (CC+AA) exhibited a mean CRP level of  $0.67 \pm 0.46$  in this cohort with low disease activity.

Furthermore, the allelic distribution of RIPK2 rs10094579 demonstrated a nearly significant association between RA patients with moderate disease activity, as measured by ALT levels ( $p = 0.065$ ), and those with high disease activity, as indicated by CRP levels ( $p = 0.058$ ).

## Discussion

The prevalence of RA is currently on the rise globally, as is its associated mortality rate. According to data from the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/snp>), the global minor allele frequency of the RIPK1 gene variants rs17548629 and rs10094579 are 0.02719 and 0.16478, respectively. These rates are considerably lower than those observed in Asian populations, where the frequencies are 0.291 and 0.2135, respectively. Limited research has explored the potential associations between RIPK1 polymorphisms and RA. Our study reveals a significant association of the RIPK1 rs17548629 with RA in the Chinese population, a finding consistent across various genetic models—additive, dominant, and co-dominant—both before and after adjusting for confounding factors. Notably, the C allele of RIPK1 rs17548629 appears to confer a protective effect (OR: 0.322, 95% CI: 0.231 to 0.449,  $p < 0.001$ ), whereas the T allele is linked to an increased risk of RA (AOR: 3.21, 95% CI: 2.29 to 4.60,  $p < 0.001$ ).

RIPK1 is a crucial regulator of necroptosis, a type of programmed cell death marked by an intense proinflammatory response.<sup>5,25</sup> Necroptosis plays a significant role in both organismal development and homeostasis and is implicated in numerous human diseases.<sup>26</sup> Notably, increased necroptotic activity has been observed in the synovium of animals with experimental autoimmune arthritis.<sup>27</sup> RIPK1 comprises a kinase domain (KD), a death domain (DD), and a RIP homotypic interaction motif (RHIM), with the DD facilitating the recruitment of various signaling complexes.<sup>28</sup> Recent studies highlight RIPK1's role in cellular content release and subsequent activation of pattern recognition receptors, which are vital for innate immune responses.<sup>29</sup> For instance, Saeki et al<sup>30</sup> elucidated the prominent role of RIPK1 in a human rheumatoid synovial cell line, while Yang et al<sup>31</sup> discovered that the rs17548629 variant introduces a new binding site for hsa-miR-1197 in RIPK1's 3'-UTR. This novel interaction appears to downregulate RIPK1 protein levels, potentially moderating inflammation and apoptosis, both critical to the pathogenesis of RA. These findings suggest that the rs17548629 variant could serve as a significant marker for RA susceptibility and early diagnosis. Furthermore, RA, akin to Behçet's disease (BD), is considered an auto-inflammatory disorder, potentially involving disruptions in the adaptive immune system and Th1/Th2 balance.<sup>32</sup> In separate research, Wu et al identified the SNP rs10094579 in RIPK2 as a susceptibility gene for BD in the Chinese Han population; however, no significant association with RA was found.<sup>33</sup>

In a study by Liu et al, it was suggested that RIPK may serve as potential markers for liver damage.<sup>34</sup> Similarly, Yao et al reported an association between the GG genotype of the RIPK1 rs2272990 polymorphism and hepatic injury.<sup>35</sup> The etiology of hepatic abnormalities in RA patients remains unclear.<sup>36</sup> Consequently, we hypothesized that RIPK gene polymorphisms might indicate organ injury in RA patients. Our study found no significant association between the two SNPs and biochemical markers such as ALT, AST, urea, glucose, or creatinine. However, a notable trend suggested a potential link between the RIPK2 rs10094579 polymorphism and ALT levels in RA patients with moderate disease activity ( $p = 0.065$ ). RIPK2, which features a carboxy-terminal caspase-activation-and-recruitment domain (CARD) and lacks a receptor-interacting protein homotypic interaction motif (RHIM),<sup>6</sup> plays a crucial role in inflammation, immune response, and liver fibrosis.<sup>37</sup> RIPK2 activation by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is significant in innate immunity.<sup>38</sup> NOD2, elevated in the knee joints of RA patients, modulates pro-inflammatory cytokine release and expression,<sup>39</sup> and is linked to inflammatory liver diseases.<sup>40</sup> Building on these findings, our future work will further investigate the relationship between RIPK2 and liver damage in RA patients, potentially illuminating the complex interactions between genetic factors, liver health, and RA pathogenesis. Pan et al have shown that RIPK2 upregulation in articular cartilage tissues correlates with osteoarthritis severity, demonstrating that RIPK2 knockdown inhibits interleukin-1 $\beta$  (IL-1 $\beta$ )-induced extracellular matrix degradation and reduces oxidative



stress.<sup>41</sup> Additionally, RIPK2 is crucial in T-cell receptor-mediated proliferation via IL-1, IL-18, and Toll-like receptors,<sup>42</sup> offering clinical implications for selecting immunosuppressive therapy in RA with moderate activity to preserve liver and kidney function. Further investigation is essential to deepen our understanding of these mechanisms.

To assess whether specific SNPs correlate directly with disease outcomes, we examined their association with disease activity indices. We found no significant relationship between the SNPs in question and the DAS28-ESR scores. Contrarily, Hao et al demonstrated a significant correlation between the IL-21 rs2055979 polymorphism and elevated DAS28 scores, particularly noting that individuals possessing the AA genotype exhibited higher scores than those with CA or CC genotypes.<sup>43</sup> Similarly, Malinowski et al differentiated patients into remission (DAS28 < 2.5) and active disease (DAS28 > 2.5) categories, observing a higher prevalence of the rs2221903 CT and CC genotypes among patients with active disease.<sup>44</sup> In our analysis, we specifically explored the relationship between the RIPK2 rs10094579 genotype and CRP levels in RA patients with low disease activity, utilizing an over-dominant model. Both CRP and ESR are established indicators of systemic inflammation.<sup>45</sup> Our findings suggest a potential linkage between the RIPK2 rs10094579 polymorphism and the inflammatory state in RA patients with low activity. Patients with the CA genotype in RIPK2 rs10094579 exhibited higher disease activity compared to those with the CC or AA genotypes. These observations imply that such patients may require intensified immunosuppressive treatment or may not be ideal candidates for the early cessation of immunosuppressants. Further research into the interaction between these genetic variants and inflammatory responses in RA patients with low disease activity is warranted.

While our study provides valuable insights, it has limitations. We analyzed only two SNPs in RIPK1 and RIPK2, and our cohort size was modest, which constrains the generalizability of our findings. Moreover, these results have not yet been validated in vivo. Nonetheless, our research lays the groundwork for early diagnosis of RA, and we plan to enlarge our cohort and perform animal model experiments to confirm these SNP variants and investigate associated signaling pathways.

## Conclusions

In summary, our study indicates that the RIPK1 rs17548629 mutation (T) significantly elevates the risk of RA in the Chinese population. Moreover, RA patients exhibiting low disease activity and carrying the CA genotype at RIPK2 rs10094579 have demonstrated elevated CRP levels compared to those with other genotypes. This observation implies that RA patients with the CA genotype at rs10094579 should exercise increased caution regarding the discontinuation of their medication. Further investigations are warranted to clarify these gene polymorphisms' roles in RA and may reveal novel therapeutic avenues for its management.

## Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Informed Consent

This study was approved by the Ethics Committee of China-Japan Friendship Hospital, and the requirement for patients to sign informed consent was waived.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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