

A20 Promoter rs5029924 Concomitant with rs2230926 and rs5029937 May Be a Prognostic Predictor for Joint Deformity or Refractory Rheumatoid Arthritis

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Background: There have been several studies regarding the susceptibility of *A20* gene SNPs (rs2230926 and rs5029937) in rheumatoid arthritis (RA). However, little is known about the association between polymorphisms in the *A20* promoter and RA. The aim of this study was to investigate the characteristics of *A20* promoter polymorphisms and the association between these polymorphisms and clinical significance in Chinese RA patients.

Methods: PCR and sequencing were used to identify *A20* gene polymorphisms in peripheral blood mononuclear cells (PBMCs) from 123 RA cases and 31 healthy individuals.

Results: Only one SNP (rs5029924) in the *A20* gene promoter was identified in RA patients and healthy individuals. 6 patients who carried heterozygous rs5029924 (3918C>T) together with heterozygous rs5029937 (11,571 G>T) and rs2230926 (12,486 T>G, Phe127Cys) suffered from joints deformity or refractory RA.

Conclusion: We reported the *A20* promoter polymorphism rs5029924 in RA patients for the first time. rs5029924 concomitant with rs2230926 and rs5029937 may be a prognostic predictor for joint deformity or refractory RA.

Keywords: *A20*, promoter, polymorphism, RA, deformity, refractory, predictor

Background

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease characterized by proliferative synovitis, the infiltration of inflammatory cells into synovial tissue, progressive joint destruction and disability. The mechanism underlying immune disorder in RA remains unclear. *A20* (*TNFAIP3*) is an important negative immunoregulatory gene located at 6q23. The intracellular ubiquitin-editing protein A20 is a key player in the negative feedback regulation of NF-kappa B signaling in response to multiple stimuli. Moreover, A20 also regulates tumor necrosis factor (TNF)-induced apoptosis.^{1,2} Many *A20* single nucleotide polymorphisms (SNPs) were found to be associated with the susceptibility to autoimmune disease.^{3,4} Variants modulate the function of an enhancer upstream of *TNFAIP3*.⁵ Little is known about the characteristics and incidence of *A20* promoter polymorphisms in Chinese RA patients.^{6,7} Therefore, we examined

A20 promoter polymorphisms and explored the association between these polymorphisms and clinical significance in Chinese RA patients.

Methods

Hospitalized adult RA patients came from the first Affiliated Hospital of Jinan University in 2015–2017. Rheumatologists diagnosed RA and these diagnoses also fulfilled the American College of Rheumatology criteria and expert opinion (1987 ACR criteria),⁸ and / or 2010 ACR/EULAR Criteria.⁹ The exclusion criteria for the RA patients was as follows: 1. Combined with other autoimmune diseases. 2. Combined with other internal medicine diseases (type 1 diabetes, type 2 diabetes, hypertension, chronic kidney disease and so on). 3. Patients with severe infections. 4. Patients with malignant tumors. 5. Smoking patients. All RA patients were assessed for clinical disease activity by a trained rheumatologist using disease activity score 28 (DAS 28). RA clinical assessment and blood sampling done were in the same day. The healthy individuals from our hospital physical examination center were in healthy status without smoking, any cancer, type 2 diabetes, hypertension, or autoimmune inflammatory disease. PCR was performed to amplify different domains of genomic DNA that encompass the promoter of the *A20* gene according to the structure of the *A20* gene from peripheral blood mononuclear cells of RA patients and healthy individuals,⁷ and the PCR products were further sequenced. The sequences were analyzed with BLAST software to identify polymorphisms or mutations in the promoter of the *A20* gene (Figure 1A, Supplemental Table 1). RF and CRP were measured by immune nephelometry, and anti-CCP antibody was measured by enzyme-linked immunosorbent assay (ELISA). All statistical analyses were performed with SPSS 23.0 software. And a *P* value of less than 0.05 was considered statistically significant. Student's *t*-test was used to compare the differences in continuous variables with normal distributions, and the Mann–Whitney *U*-test was used for continuous variables with non-normal distributions between two groups. Pearson's Chi square test was used to compare the distribution of genotypes and alleles between RA group and healthy control group. In cases in which the genotype had a frequency of less than 1, Fisher's exact test was applied. Data are expressed as mean±SD unless otherwise specified.

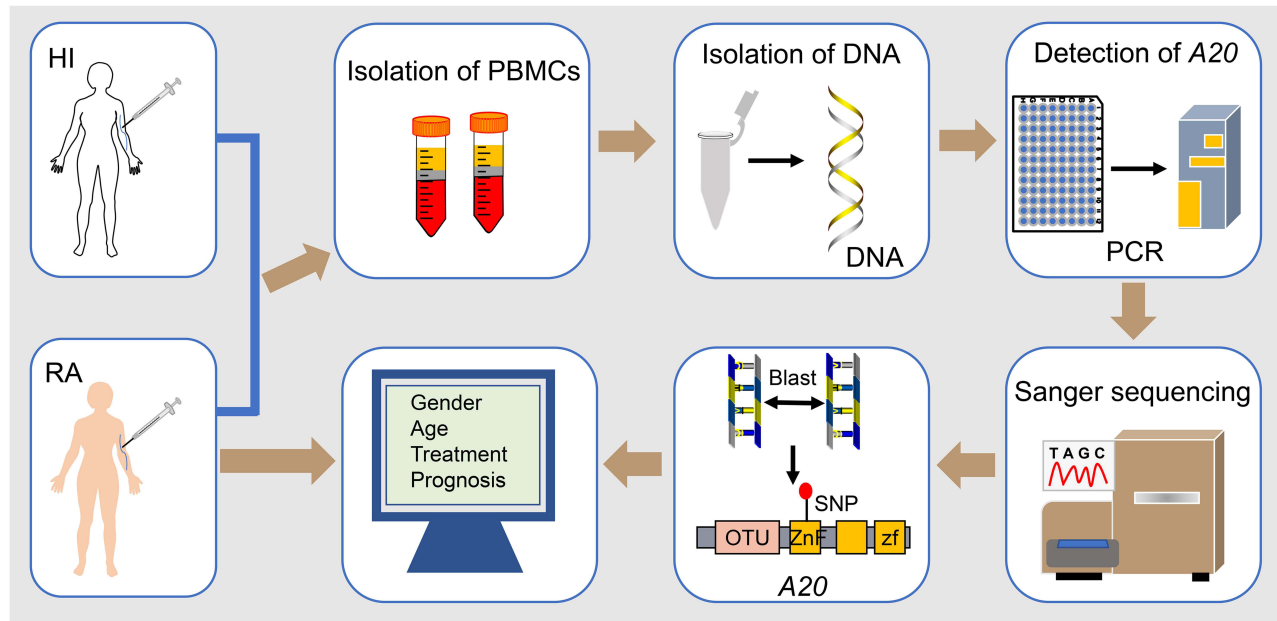
Results and Discussion

This study included samples from 123 RA cases and 31 healthy individuals who provided written informed consent (Supplemental Table 2). There were no significant differences in the distributions of the genders and ages between the cases and healthy individuals (*P* > 0.05). All RA patients accepted glucocorticoids, disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate (MTX), leflunomide (LEF) as initial treatment. Anti-TNF- α antibody (Etanercept, Infliximab or Adalimumab) or Tocilizumab was combined when efficacy was poor.

Only one SNP, rs5029924 (3918C>T), was identified in six patients with RA and three healthy individuals (Figure 1B), revealing that the *A20* promoter is more highly conserved than its exons and introns.¹⁰ Moreover, there have been no reports on the frequency of rs5029924 in RA patients. In this study, we found that the frequency of the rs5029924 CT genotype was 4.88% in RA patients, and it appeared to be higher (9.68%) in healthy controls; however, there was no statistically significant difference between the two populations (*P* = 0.555) (Table 1), which may be due to the limited number of samples in this study. Thus, the association between rs5029924 and RA requires further investigation with a larger cohort of samples.

Interestingly, the six patients carrying heterozygous rs5029924 (CT genotype) also harbored heterozygous rs5029937 (11,571 G>T) (GT genotype) and rs2230926 (12,486 T>G, Phe127Cys) (TG genotype) (Figure 1B), which were previously demonstrated to be risk SNPs for RA.³ Moreover, the remaining 117 cases without heterozygous rs5029924 were not heterozygous for rs5029937 and rs2230926. They all carried rs5029924 (CC genotype), rs5029937 (GG genotype) and rs2230926 (TT genotype). This finding may indicate that there is genetic linkage between rs5029924, rs5029937, and rs2230926. Although rs5029924 was located in the promoter, it may affect *A20* expression. It has been reported that rs5029924 affects *A20* gene promoter activity, resulting in significantly decreased *A20* protein expression.¹¹ rs5029924, rs5029937 and rs2230926 were also identified simultaneously in a Sézary syndrome case.¹² rs5029937 and rs2230926 are associated with the increased risk of RA.¹³ The functional prediction of rs5029924 in the *A20* gene is shown in Supplemental Table 3. In addition, rs2230926 (12,486 T>G, Phe127Cys) is in exon 3 of *A20*. The risk allele (Cys127) leads to reduced inhibition of NF- κ B activation or reduced *A20* mRNA levels.¹⁴

A



B

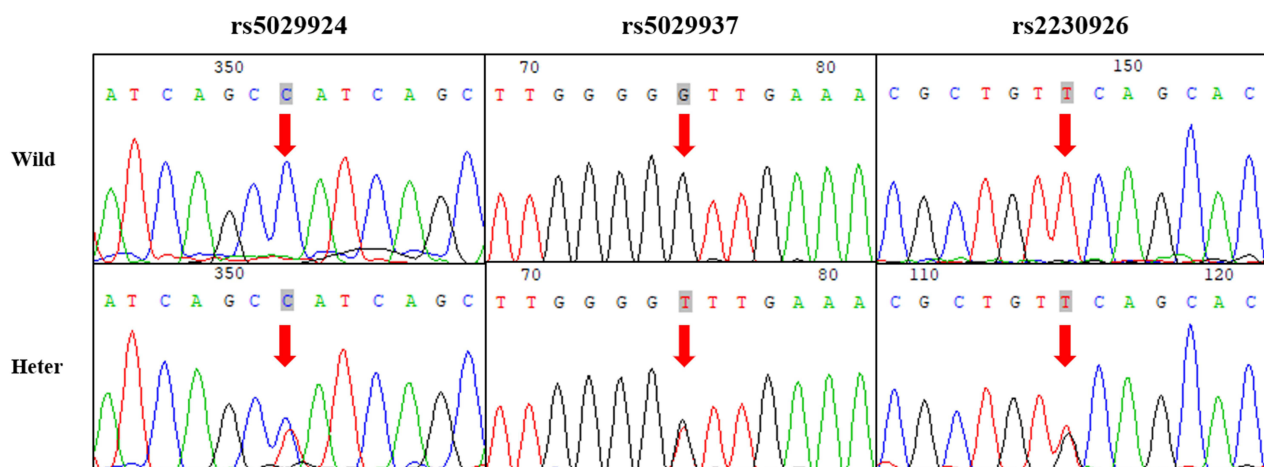


Figure 1 A20 rs5029924, rs5029937 and rs2230926 identified in RA patients. (A) Experimental procedure in this study; (B) rs5029924, rs5029937 and rs2230926 identification in a RA patient. The arrows indicate the site of nucleotide change. Wild: Wild-type; Heter: Heterozygous.

In addition, we found that six RA patients who carried heterozygous rs5029924 together with heterozygous rs5029937 and rs2230926 suffered from severe refractory disease or had poor prognosis (Table 2). The first patient suffered obvious joints deformities in multiple joints of the hands and knees. He was diagnosed with lung adenocarcinoma after suffering from RA for ten years and died two years after the cancer diagnosis. Most of them suffered from chronic, irreversible joints deformities, and demonstrated poor response to glucocorticoids, disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate (MTX), leflunomide (LEF), and needed combination of biologic DMARDs, such as anti-TNF- α antibody, or Tocilizumab. Overall, these preliminary data suggest that rs5029924, rs5029937, and rs2230926 in *A20* may be related to poor clinical outcome for RA, and these SNPs might be a prognostic biomarker for joint deformity or refractory disease. rs5029924, rs5029937, and rs2230926 may be involved in *A20*-mediated inflammatory pathways. Undoubtedly, identifying

Table 1 Frequencies of the rs5029924, rs2230926, and rs5029937 Alleles and Genotypes in RA Patients and Healthy Individuals

SNPs	Genotype Frequency n (%)			Allele Frequency n (%)	
	Major Homozygote	Heterozygote	Minor Homozygote	Major Allele	Minor Allele
rs5029924	CC	CT	TT	C	T
RA (n=123, %)	117 (95.1)	6 (4.9)	0 (0)	240 (97.6)	6 (2.4)
Controls (n=31, %)	28 (90.3)	3 (9.7)	0 (0)	59 (95.2)	3 (4.8)
p-value	-	0.555	-	-	0.561
rs5029937	GG	GT	TT	G	T
RA (n=123, %)	117 (95.1)	6 (4.9)	0 (0)	240 (97.6)	6 (2.4)
Controls (n=31, %)	28 (90.3)	3 (9.7)	0 (0)	59 (95.2)	3 (4.8)
p-value	-	0.555	-	-	0.561
rs2230926	TT	TG	GG	T	G
RA (n=123, %)	117 (95.1)	6 (4.9)	0 (0)	240 (97.6)	6 (2.4)
Controls (n=31, %)	28 (90.3)	3 (9.7)	0 (0)	59 (95.2)	3 (4.8)
p-value	-	0.555	-	-	0.561

Abbreviation: SNPs, nucleotide polymorphisms.

Table 2 Clinical Characteristics of RA Patients Heterozygous for rs5029924, rs2230926 and rs5029937

Patients	Gender	Age(y)	Treatment	Prognosis
P1	Male	72	LEF, MTX	Joints deformities, poor response to DMARDs and died from lung cancer
P2	Female	63	LEF, MTX	Joints deformities, poor response to DMARDs
P3	Female	52	LEF, MTX, Etanercept	Joints deformities, poor response to DMARDs and Etanercept
P4	Female	46	LEF, MTX, Etanercept	Joints deformities, poor response to DMARDs and Etanercept.
P5	Female	54	LEF, MTX, Infliximab, Tocilizumab	Poor response to DMARDs, Infliximab, and Tocilizumab
P6	Female	51	LEF, MTX,	Joints deformities, poor response to DMARDs

Abbreviations: DMARDs, Disease modifying anti-rheumatic drugs; LEF, leflunomide; MTX: methotrexate.

novel genetic risk factors is imperative to better understand RA pathogenesis. However, further investigation is needed using a large cohort of samples to confirm this finding.

Conclusions

In conclusion, we characterized polymorphisms in the *A20* promoter and for the first time identified the *A20* promoter polymorphism rs5029924 in Chinese RA patients. rs5029924 concomitant with rs2230926 and rs5029937 may be a prognostic biomarker for joint deformity or refractory RA. Further studies are required to confirm the frequency and function of rs5029924.

Abbreviations

anti-CCP, anti-cyclic citrullinated peptide antibody; CRP, C reactive protein; DAS 28, disease activity score 28; DMARDs, Disease modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate; LEF, leflunomide; MTX, methotrexate; PBMCs, Peripheral blood mononuclear cells; RA, Rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; TNFAIP3 (*A20*), tumor necrosis factor α induced protein-3.

Data Sharing Statement

All supporting data are included in the manuscript and supplemental files. Additional data are available upon reasonable request to the corresponding author.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of First Affiliated Hospital, Jinan University. All participants provided written informed consent. Our study complied with the Declaration of Helsinki.

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.

Funding

This study was supported by grants from the National Natural Science Foundation of China (Nos. 82270140), the Natural Science Foundation of Guangdong province (Nos.2017A030310456), the Funding by Science and Technology Projects in Guangzhou (Nos.2024A03J0819, 2024A03J0824), the Medical Scientific Research Foundation of Guangdong Province (Nos. A2018271 and A2019221), and the Science Research and Cultivate Foundation from the First Clinical Medical College of Jinan University (Nos. 2016320, 2017105, and 2018108).

Disclosure

The authors declare that they have no competing interests.

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