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RESEARCH ARTICLE

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A novel *CD209* polymorphism is associated with rheumatoid arthritis patients in Taiwan

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Abstract

Single nucleotide polymorphisms (SNPs) in the promoter region of *CD209* (cluster of differentiation 209) may influence expression levels, and higher expression of *CD209* on immune cells correlate with severity of cartilage destruction in patients with rheumatoid arthritis (RA). Due to the lack of a comprehensive study, this study aimed to investigate the *CD209* promoter variants and haplotypes in a Taiwanese population and the association with RA development. Deoxyribonucleic acid (DNA) of peripheral blood mononuclear cells from 126 RA patients and 124 healthy controls was purified, and the *CD209* gene promoter was amplified by polymerase chain reaction and analyzed by Sanger sequencing. Results showed that a novel variant −96C>A polymorphism in *CD209* promoter was identified in the Taiwanese population, and the frequency was significantly higher in RA patients than in controls (11.51% vs. 2.42%, P < .0001). The odds ratio (OR) for the development of RA was 5.88 (95% CI 2.35–14.74, P < .0001). Other known variants were also evaluated; for instance, −1180 T/T (rs7359874) was increased in RA patients, and the OR for the development of RA was 3.26, 95% CI 0.85–12.52, P = .07). Besides, the haplotype frequencies were calculated; −1180A-939C-871 T-336 T-139 T-96A and −1180 T-939 T-871C-336 T-139C-96A were increased in RA patients (P = .004 and 0.05, respectively). In summary, CD209-96A variant could be an important factor for the development of RA in the Taiwanese population.

KEYWORDS
autoimmunity, CD209 polymorphism, rheumatoid arthritis

Hua-Chen Chan and Shu-Chen Wang are contributed equally.

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that associates with multiple heritable genetic factors. Although genetic variations of the *major histocompatibility complex* (*MHC*) contribute approximately 60% of susceptibility, recently, a genome-wide association study revealed that about 100 non-MHC genes are associated with RA as well.[1,2] Other linkage studies such as Suzuki et al[3] also demonstrated that the *peptidylarginine deiminase 4 (PADI4)* genetic polymorphism associates with the susceptibility to RA. However, though significant in Japanese and Mexican populations, the *PADI4* functional haplotype was not associated with RA in the United Kingdom.[4-6] Similarly, *protein tyrosine phosphatase non-receptor 22* polymorphisms were significantly associated with RA in Europe, but not in Latin American.[7,8] Considering the ethnicity factor, the RA-risk-associated genomic polymorphisms in Taiwan must be evaluated.

The etiology of RA is complicated. Previous studies have suggested that dendritic cells and macrophages play the central roles for triggering T- and B-cell activation, which is of great importance to assess RA disease onset.[9,10] In the synovium of PA patients, the infiltration of monocyte-derived macrophages or the recruitment of synovial dendritic cells can be observed. These cells express the C-type lectin adhesion molecule to interact with T cells for releasing pro-inflammatory mediators.[11,12] The cluster of differentiation 209 (CD209), also known as dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), is a C-type lectin expressed on dendritic cells and macrophages.[13,14] In the synovium of RA patients, CD209 is highly expressed on synovial CD68+ macrophages and co-localized with intracellular adhesion molecule (ICAM)-3-positive resting T cells; in contrast, these phenomena cannot be found in the synovium of osteoarthritis or trauma patients.[12] CD209 regulates the monocyte-induced T-cell activation in the RA synovium and modulates adaptive immune responses to stimulate osteoclastogenesis and bone structural damage in RA,[15] so increased expression levels of CD209 might play an essential role in RA patients' pathogenesis.

Several studies proposed that single nucleotide polymorphisms (SNPs) of CD209 promotor sequence are associated with the susceptibility to viral infections, such as dengue virus, human immunodeficiency virus type 1, and hepatitis C virus.[16-18] These infections may also contribute to the development of RA.[19] In contrast to its pathogenic role in infectious diseases, recent studies revealed that two SNPs in the promoter region of CD209 (rs4804803 and rs735239) showed protective effects against the development of type 1 diabetes, a kind of immune-related disease.[20] The exact mechanism is still obscure.

It remains controversial as to whether polymorphisms in the promoter of *CD209* are associated with RA. Besides, the CD209 promoter variants and haplotypes in the Taiwanese population are undetermined. This study is, therefore, aimed at analyzing the full promoter sequence of *CD209* by Sanger sequencing and in the associations between *CD209* variants and RA development.

2 | MATERIALS AND METHODS

2.1 | Study subjects

The Institutional Review Board of Kaohsiung Medical University Hospital (KMUH) approved this study. A total of 126 RA patients were diagnosed according to the 2010 ACR/EULAR (American College of Rheumatology/European League Against Rheumatism) RA classification criteria[21] and enrolled, with a control population being comprised of 124 healthy individuals.

2.2 | Preparation of genomic DNA

According to the manufacturer's instructions, peripheral blood mononuclear cells (PBMCs) of RA patients and controls were separated by a density gradient centrifugation method (Ficoll-paque PLUS; GE Healthcare Life Sciences). Genetic DNA was extracted from PBMCs by using the Gentra Puregeae Blood kit (Qiagen).

2.3 | Polymerase chain reaction for CD209 promotor region

Polymerase chain reaction (PCR) was performed by using the Taq PCR Master Mix (Hoffmann-La-Roche). Two regions of the *CD209* promoter were amplified. The primer sequences were listed as follows: the first pair primers (forward: 5′-CACATCATCTCATCTGGAC-3′; reverse: 5′-GATTGGAATACTATACAGC-3′); the second pair primers (forward 5′-GTTAGCTAAACTTGCAGTGC-3′; reverse 5′-CCAC AGCTTTTATTTCCCAC-3′). PCR protocol was 95°C for 3 min for DNA denaturation; 35 cycles of amplification (95°C for 45 s, 60°C for 1 min, and 72°C for 1 min); and a final extension phase at 72°C for 7 min.

2.4 | DNA sequencing

PCR-sequencing was performed by using the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) according to the manufacturer's instruction. The PCR program was 96°C for 1 min for initial denaturation; 25 cycles of amplification composed of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. In the end, PCR amplified products were held at 4°C and then DNA products were purified by using the Sequencing Purification & Clean-Up Kit (ThermoFisher Scientific). Sequences were analyzed by the Applied Biosystems 3730xl DNA Analyzer (ThermoFisher Scientific). Electropherogram reads were assembled and analyzed with the Variant Analysis software (ThermoFisher Scientific).

2.5 | Statistical analysis

Hardy–Weinberg equilibrium was calculated using standard observed-expected chi-squared (χ^2) analysis with a threshold of 0.05 at each

polymorphic site. Allele frequencies and genotypes were determined by counting, and the most common allele in the control group was assigned as the reference category. The chi-squared test or Fisher exact test was applied to evaluate the statistical significance for all comparisons. The estimated haplotype frequencies were determined by the Haploview software (Broad Institute of Massachusetts Institute of Technology and Harvard University). Haplotype blocks were constructed based on D' in healthy control subjects, with a D' of >0.8 for pairs of marker representing a haplotype block. The risk association between each allele and disease was determined by odds ratio (OR) with a 95% confidence interval (CI). All statistics were conducted using the SSPS statistical software (version 20.0, SPSS Inc.), with all tests considered to be statistically significant when $P < .05$.

3 | RESULTS

3.1 | Characteristics of the study participants

A total of 126 patients with RA were enrolled in this study, 20 subjects were male, and 106 were female. Their average age was 61.83 ± 12.94 years. One-hundred and twenty-four gender-compatible healthy controls were recruited, including 31 males and 93 females with mean age of 59.45 ± 7.86. There were no significant differences in gender and age between RA patients and healthy controls. The mean onset age was 47.39 ± 14.07, and their disease duration was 14.79 ± 10.17 on average (Table 1). Results of biochemical examinations indicated that 83.90% of them had rheumatoid factors, and 81.19% of them had anti-cyclic citrullinated peptide antibodies. Besides, the physical examination revealed that other signs such as rheumatoid nodules (25.42%), cutaneous vasculitis (2.54%), and neuropathy (9.32%) were found in these patients.

3.2 | SNPs of the CD209 promoter region

After direct sequencing for the *CD209* promoter region in RA patients and healthy controls, 6 SNPs were found including rs7359874 (−1180A>T), rs735240 (−939C>T), rs735239 (−871 T > C), rs4804803 (−336 T > C), rs2287886 (−139 T > C), and rs558555834 (−96C>A). In the SNP database of the National Center for Biotechnology Information (NCBI), the rs558555834 is a C to T polymorphism at the −96 promoter region of *CD209*. However, results showed that the −96 of *CD209* in the Taiwanese population was C to A polymorphism (Figure 1). The allele and genotype frequencies of the *CD209* promoter SNPs in RA patients and healthy controls are shown in Table 2. Chi-square for Hardy–Weinberg equilibrium were tested with the results being 0.61 (−1180A>T), 0.52 (−939C>T), 0.35 (871 T > C), 0.84 (−336 T > C), 0.90 (−139 T > C), and 0.78 (−96C>A), respectively, indicating that the genotype distributions of these polymorphisms were compatible with Hardy-Weinberg equilibrium. The map of the *CD209* gene and linkage disequilibrium among SNPs is shown in Figure 2.

There was no significant difference in the genotype and allele frequencies of rs735240 (−939 T), rs735239 (−871C), rs4804803 (−336C), and rs2287886 (−139C) between RA patients and healthy controls (Table 2), although the −1180 T/T (rs7359874) genotype frequency was higher in RA patients than healthy controls (7.14% vs. 2.42%, OR =3.26, 95%CI =0.85–12.52, P = .07) (Table 2).

The genotype distribution of rs558555834 (−96C/A) was significantly increased in RA patients as compared to healthy controls (23.02% vs. 4.84%, OR =5.88, 95% CI =2.35–14.74, p <.0001). The association

TABLE 1 Characteristics of RA patients and healthy controls

	NC (n = 124)	RA (n = 126)
Gender (male/female)	31/93	20/106
Age (years)	59.45 ± 7.86	61.83 ± 12.94
Onset age (years)	N/A	47.39 ± 14.07
Disease duration (years)	N/A	14.79 ± 10.17
Rheumatoid factor (%)	N/A	83.90
Anti-cyclic citrullinated peptide antibodies (%)	N/A	81.19
Rheumatoid nodules (%)	N/A	25.42
Cutaneous vasculitis (%)	N/A	2.54
Neuropathy (%)	N/A	9.32

N/A, not applicable.

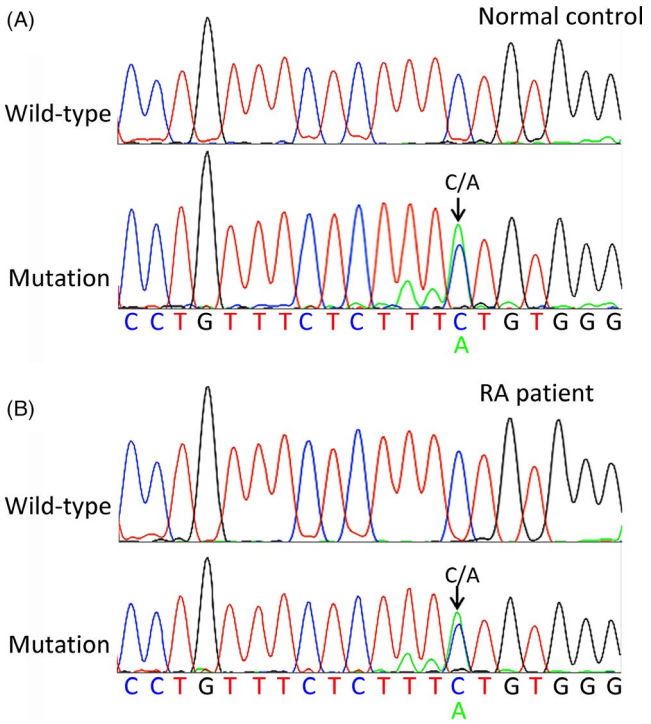

FIGURE 1 Direct sequence analysis of rs558555834 showed heterogeneous C to A variation. The genomic DNA was isolated from PBMC in 126 RA patients and 124 healthy controls. Direct sequencing was performed to analyze the polymorphisms in the CD209 promoter region. A segment of nucleotide 4915–4934 sequence located in CD209 gene promoter indicates wild type or C/A mutation in (A) healthy control, or in (B) RA patient

TABLE 2 Genotype and allele frequencies of CD209 in RA and healthy controls

SNP	RA (n = 126), %	NC (n = 124), %	OR (95%, CI)	P value
rs7359874 (−1180A>T)				
A/A	70 (55.56)	76 (61.29)		
A/T	47 (37.30)	45 (36.29)	1.13 (0.67–1.91)	.69
T/T	9 (7.14)	3 (2.42)	3.26 (0.85–12.52)	.07
A/A versus A/T + T/T			1.27 (0.77–2.10)	.36
A/A + A/T versus T/T			3.10 (0.82–11.74)	.08
A	187 (74.21)	197 (79.44)		
T	65 (25.79)	51 (20.56)	1.34 (0.88–2.04)	.17
rs735240 (−939C>T)				
C/C	71 (56.35)	77 (62.10)		
C/T	47 (37.30)	44 (35.48)	1.16 (0.69–1.95)	.60
T/T	8 (6.35)	3 (2.42)	2.89 (0.74–11.33)	.11
C/C versus C/T + T/T			1.27 (0.77–2.10)	.36
C/C + C/T versus T/T			2.73 (0.71–10.56)	.13
C	189 (75.00)	198 (79.84)		
T	63 (25.00)	50 (20.16)	1.32 (0.87–2.01)	.19
rs735239 (−871 T > C)				
T/T	84 (66.67)	87 (70.16)		
T/C	37 (29.37)	37 (29.84)	1.04 (0.6–1.79)	.89
C/C	5 (3.97)	0 (0.00)		
T/T versus T/C + C/C			1.18 (0.69–2.01)	.55
T	205 (81.35)	211 (85.08)		
C	47 (18.65)	37 (14.92)	1.31 (0.82–2.10)	.26
rs4804803 (−336 T > C)				
T/T	109 (86.51)	101 (81.45)		
T/C	16 (12.70)	22 (17.74)	0.67 (0.34–1.36)	.27
C/C	1 (0.79)	1 (0.81)	0.93 (0.06–15.01)	.96
T/T versus T/C + C/C			0.68 (0.35–1.36)	.28
T/T + T/C versus C/C			0.98 (0.06–15.91)	.99
T	234 (92.86)	224 (90.32)		
C	18 (7.14)	24 (9.68)	0.72 (0.38–1.36)	.31
rs2287886 (−139 T > C)				
T/T	61 (48.41)	59 (47.58)		
T/C	51 (40.48)	56 (45.16)	0.88 (0.52–1.48)	.63
C/C	14 (11.11)	9 (7.26)	1.50 (0.61–3.74)	.38
T/T versus T/C + C/C			0.97 (0.59–1.59)	.90
T/T + T/C versus C/C			1.6 (0.66–3.84)	.29
T	173 (68.65)	174 (70.16)		
C	79 (31.35)	74 (29.84)	1.07 (0.73–1.57)	.71
rs558555834 (−96C>A)				
C/C	97 (76.98)	118 (95.16)		
C/A	29 (23.02)	6 (4.84)	5.88 (2.35–14.74)	**<.0001**
C/C versus C/A + A/A			5.88 (2.35–14.47)	**<.0001**
C	223 (88.49)	242 (97.58)		
A	29 (11.51)	6 (2.42)	5.25 (2.14–12.87)	**<.0001**

P value was determined by using the chi-squared test or Fisher's exact test. Hardy Weinberg equilibrium (HWE).

Abbreviations: NC, healthy control; RA, rheumatoid arthritis.

Significant values are indicated in bold.

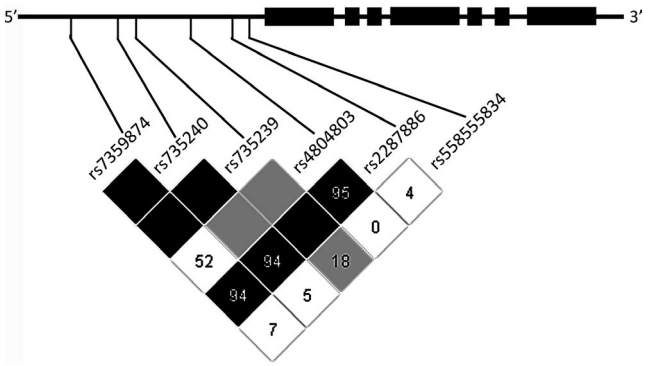

FIGURE 2 Map of the CD209 gene promoter and linkage disequilibrium among SNPs. Values represent D's. Black areas denote haplotype blocks based on *D′* values

TABLE 3 CD209 haplotype frequencies in RA patients and healthy controls

SNP position	RA	NC	P value
−1180A−939C−871 T−336 T−139 T−96C	0.60	0.67	.10
−1180 T−939 T−871C−336 T−139C−96C	0.17	0.14	.49
−1180A−939C−871 T−336C−139C−96C	0.06	0.08	.23
−1180 T−939 T−871 T−336 T−139C−96C	0.05	0.05	.97
−1180A−939C−871 T−336 T−139 T−96A	0.07	0.02	**.004**
−1180 T−939 T−871C−336 T−139C−96A	0.02	0.002	**.05**

Significant values are indicated in bold.

of −96A with RA patients was in a dominant model (C/C vs. C/A + A/A, OR =5.88, 95% CI =2.35–14.47, *P* <.0001). The allele frequency of −96A was also significantly higher in RA patients than in healthy controls (11.51% vs. 2.42%, OR =5.25, 95%CI =2.14–12.87, *P* <.0001) (Table 2).

3.3 | CD209 haplotype frequencies

The most common CD209 haplotype in Taiwan was −1180A-939C-871 T-336 T-139 T-96C (Table 3). The frequency of −1180A-939C-871 T-336 T-139 T-96A haplotype was significantly higher in RA patients than in healthy controls (0.07 vs. 0.02, *P* =.004), and the −1180 T-939 T-871C-336 T-139C-96A haplotype frequency also tended to be increased in RA patients compared with healthy controls (0.02 vs. 0.002, P =.05) (Table 3). Additionally, the analysis of combined allele frequencies of rs7359874 (−1180A/T) and rs558555834 (−96C/A) revealed associations of two haplotypes with RA. The −1180A-96A haplotype was significantly more frequent in RA patients than in healthy controls (12.9% vs. 3.6%, P =.0005).

4 | DISCUSSION

In this study, a novel *CD209* gene promoter polymorphisms −96C>A was identified from a Taiwanese population; −96C>A SNP carriers showed an increased risk of RA development.

CD209 is a type II transmembrane protein with an external mannose-binding C-type lectin domain that can bind the glycan-rich HIV-1 envelope.[22] Recently, Rocio et al reported that a haplotype of *CD209* promoter, rs4804803 (−336 T)-rs735239 (−871G)-rs735240 (−939 T), is associated with HIV-1 infection. They cloned the *CD209* promoter variants and evaluated the promoter activities in a cell model, with the results indicating that CD209 expression was decreased in transfected cells.[16] Another study provided similar evidence to demonstrate that increasing the frequencies of haplotype rs735240 (−939A)-rs735239 (−871C) in the *CD209* promoter was associated with HCV infection.[18] These data suggested that CD209 signaling in dendritic cells might contribute to preventing viral infection.

Dendritic cells are professional antigen-presenting cells that bridge innate and adaptive immunity. They play a pivotal role in the differentiation of naive to effector CD4+ T cells, cross-priming CD8+ T cells, and promoting B cell antibody production.[23] As a receptor expressed on DCs, CD209 contributes to stabilizing the interaction between dendritic cells and T cells.[24] *CD209* promoter polymorphisms increase the susceptibility to autoimmunity, which has been reported to correlate with RA and type 1 diabetes.[20,25]

Rafael et al showed a gender-specific difference of $CD209_{rs4804803G}$ (−336C) between women and men, which indicated that carriers of $CD209_{rs4804803G}$ (−336C) had a decreased risk of developing RA in male. The protective effect of carriers with $CD209_{rs4804803G}$ (−336C) cannot be found in females. On the contrary, Caucasian males carrying $CD209_{rs2287886A}$ (−139 T) have a higher risk for RA. The association of $CD209_{rs2287886A}$ (−139 T) with RA was not significant in females[25]; however, our data showed that rs735239 (−871 T > C), rs4804803 (−336 T > C), and rs2287886 (−139 T > C) did not associate with RA patients in Taiwan. Rebeca et al also demonstrated no significant difference of rs4804803 (−336 T > C) between RA patients and healthy controls of Spanish.[26]

In this study, it was found that rs7359874 (−1180 T/T) and rs558555834 (−96C/A) genotypes were increased in RA patients. Additionally, rs558555834 C to A is a novel polymorphism in Taiwan. Based on Gene Transcription Regulation Database published in 2019, rs7359874 (−1180A>T) is located on the CCCTC-binding factor (CTCF) binding site, and rs558555834 (−96C>A) is located on or near transcription factor-binding sites including SPI-1, IRF1, and NF-kB.[27] One recent study demonstrated that CTCF contributes to maintaining the homeostasis of dendritic cell function in the immune system,[28] and in another study, polymorphism in the NF-κB binding site of the *CD209* promoter region could affect the transcription activity leading to decreased CD209 expression.[29] Additionally, CD209 has been demonstrated to drive follicular T helper cell differentiation to secrete interleukin-27 (IL-27). *In vitro* evidence suggested that IL-27 had a pro-inflammatory effect of increasing pro-inflammatory cytokines release from fibroblast-like synoviocytes.[30] However, substantial *in vivo* evidence also supports a profound anti-inflammatory role for IL-27; for example, intraperitoneal injection of IL-27 to collagen-induced arthritis mice attenuates joint inflammation, erosion, and synovial hyperplasia.[31,32]

The results of Ingenuity Pathway Analysis, Genemania, and String showed that CD209 was majorly located on plasma membrane (Figure S1), and from there, it was found that decrease of CD209 downstream induces high levels of chemokine C-C motif ligand 3 and 4 (CCL3 and CCL4). A study showed that high level of CCL4 is associated with susceptibility of RA,[33] and another study found that using CCL3 null mice model showed that CCL3 is essential for joint inflammation and destruction in mice.[34] Furthermore, our study found protein–protein interaction between CD209 and other subcellular molecules such as Dectin-1, also known as CLEC7A and CLEC4 M (Figure S1 and S2). The interaction between CD209 and Dectin-1 triggered the COX-2 signaling pathway leading to generate prostaglandin E2 from arachidonic acid hydrolysis, which promoted dendritic cell maturation.[35] One study demonstrated that immature dendritic cells were accumulated in rheumatoid synovium and were attracted into RA synovium in response to CCL20 expressed on lining layer leading to local maturation of dendritic cells and, in turn, increased inflammation in synovium.[36] Here, we also found CD209 might interact with CLEC4 M, also known as CD209L, which was majorly expressed on the endothelium of the liver, lymph nodes, and placenta. In an arthritic DA rat model, the results showed that *CLEC4 M* gene was higher in arthritis-susceptible rats than in controls[37]; however, data on the role of CLEC4 M in RA development is scarce. Based on the String database, we found the interaction between CD209 and Butyrophilin subfamily 2 member A1 (BTN2A) (Figure S3). BTN2A has been shown to bind to CD209.[38] The signaling of BTN2A has been proved to reduce the expression of various cytokines, including IL-2 and IFN-γ.[39] It was described that elevation of IL-2 and IFN-γ involved in pathogenesis of RA.[40,41] In this study, we found carriers of rs558555834 (−96A) or −1180A-939C-871 T-336 T-139 T-96A and −1180 T-939 T-871C-336 T-139C-96A haplotypes were significantly increased in RA patients, which might compromise CD209 signaling properties.

The frequencies of −1180A-939C-871 T-336 T-139 T-96A and −1180 T-939 T-871C-336 T-139C-96A haplotypes are significantly increased in RA patients. A pathogenic haplotype with −1180A-96A has 4.9-fold higher risk in RA patients than in healthy controls, while a haplotype with −1180 T-96A has 7.03-fold higher risk in RA patients than in healthy controls (data not shown). The odds ratios were around five to sevenfold increase in patients with these two haplotypes, suggesting that these two haplotypes might contribute to the development of RA.

For these refractory RA patients, biologics or targeted small molecules could be prescribed.

In this study, there was no significant difference in the percentage of using biologics (TNF-alpha blockers, IL-6 inhibitor—Actemra, Inhibitor of T-cell co-stimulation—Orencia, and B-cell inhibitor—Mabthera) or tyrosine kinase inhibitors in RA patients with or without polymorphisms. This study also demonstrated that CD209 polymorphisms did not associate with surgery for RA patients' progressive joint destruction. Therefore, the *CD209* polymorphisms could not be related to the disease activity and severity of RA patients.

In conclusion, a novel *CD209* polymorphism (−96A) in a Taiwanese population was found. Carrying −96A polymorphism showed an increased risk of RA. We also found that the −1180A-939C-871 T-336 T-139 T-96A and −1180 T-939 T-871C-336 T-139C-96A haplotypes are associated with susceptibility to RA in Taiwan. To our knowledge, this is the first association study showing that *CD209* −96A is involved in developing RA.

CONFLICTS OF INTEREST

The authors have not declared any conflicts of interest.

AUTHOR CONTRIBUTIONS

H-C Chan, S-C Wang, and J-H Yen involved in conceptualization; H-C Chan, S-C Wang, and J-H Yen involved in methodology; C-H Lin, Y-Z Lin and R-N Li involved in software; C-H Lin and Y-Z Lin involved in validation; H-C Chan and Y-Z Lin involved in formal analysis; H-C Chan, S-C Wang, and J-H Yen involved in investigation; H-C Chan, S-C Wang, and J-H Yen involved in resources; Y-Z Lin and C-H Lin involved in data curation; H-C Chan involved in writing—original draft preparation; J-H Yen; visualization, H-C Chan involved in writing—review and editing; J-H Yen involved in supervision; C-H Lin involved in project administration; H-C Chan, S-C Wang, and J-H Yen involved in funding acquisition.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The clinical results are not publicly available due to ethical restrictions. The clinical findings of this study are available on request from the corresponding. Figure S1-S3 are available at the websites of https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis, https://genemania.org, and https://string-db.org

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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