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ORIGINAL ARTICLE

Association of tri-nucleotide (CAG and GGC) repeat polymorphism of androgen receptor gene in Taiwanese women with refractory or remission rheumatoid arthritis

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Abstract We investigated the relationship between CAG and GGC repeat polymorphism of the androgen receptor (AR) gene and rheumatoid arthritis (RA) in female patients with different disease subtypes. This case-control study enrolled 215 women in three groups: RA patients refractory to standardized therapy (n=51); RA patients at complete remission phase (n=60); and healthy controls (n=104). CAG and GGC repeat lengths were determined by automated fluorescence-based DNA fragment-sizing method. Demographic data, allele lengths, allele distribution, and zygosity status of CAG/GGC repeats were assessed for the three groups. Refractory RA patients tend to have a significantly younger onset age of RA and more elevated erythrocyte sedimentation rates than do remission RA patients. Mean and median values of CAG and GGC repeat lengths are similar in both RA and control patients. However, RA patients harboring any long CAG alleles with more than 23 repeats had an increased risk of a refractory course, whereas differences in risk were not observed between these patients and RA subtypes harboring any long GGC alleles with more than 16 repeats. In addition, the homozygous frequency of CAG but not GGC alleles was lower in refractory RA than in remission RA patients or in controls (p=0.042). Neither CAG nor GGC repeat lengths had a significant relationship with rheumatoid factor reactivity. Our observations indicate that short CAG repeats of the AR gene with higher transactivation activity may have protective effects against refractory course of RA development and that homozygous frequency of CAG alleles may be involved in the disease remission subtype. In contrast, lack of association of GGC polymorphism and RA was also observed. Together, these data imply that CAG but not GGC alleles in the AR polymorphism may play an important role in modulating the disease pattern of RA among Taiwanese women.

Keywords Androgen receptor · Rheumatoid arthritis · Tri-nucleotide repeat polymorphism

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Introduction

Rheumatoid arthritis (RA) is a complex genetic disease with phenotype variations in terms of clinical presentation

at disease onset, as well as the pattern of disease progression and severity [1, 2]. While women have an approximately two to fourfold increased risk of the disease compared to men, the exact mechanism underlying this gender difference in RA remains unclear. In general, immune reactivity is more pronounced in women than men, and women have a higher incidence rate of autoimmune disease compared to men. A variety of evidence suggests that sex hormones may play important roles in regulating autoimmunity response and could affect onset and severity of immune-mediated pathological conditions by modulating lymphocytes at all stages of life [3, 4].

Low androgen levels and a reduced androgen–estrogen ratio have been identified in body fluids of RA patients [5], and it has been noted that androgen concentrations are lower than expected before RA onset in women [6]. Several reports have shown that sex differences in the pattern of RA may be related to higher testosterone levels in men through suppression of both cell-mediated and humeral immune responses by androgens [5, 7, 8]. It has been reported that androgen ablation therapy in prostate cancer may be a risk factor for development of RA [9], but on the other hand, androgen replacement therapy may improve clinical and immunological parameters associated with RA [8, 10]. In addition, disease severity of RA has been correlated with androgen levels [11], which were significantly lower in seropositive RA patients than in seronegative RA patients [12, 13]. Taken together, these reports suggest that decreased levels of androgens may be associated with pathogenesis or severity of RA.

The androgen signal pathway is mediated by the androgen receptor (AR), which is a member of the steroid hormone superfamily and known to activate a variety of androgen-targeted gene expressions in androgen responsive tissues [14, 15]. The human AR gene located in chromosome X contains two uninterrupted polymorphic CAG and GGC repeats in the coding region of exon 1. In vitro and in vivo studies have shown that the length of CAG and GGC repeats in the AR gene correlates inversely with AR transactivation function [16–18]. The numbers of trinucleotide repeats appear to be related to the age of onset or severity of several diseases such as spinal and bulbar muscular atrophy and coronary artery disease [19, 20]. Moreover, women with relatively longer CAG repeats in the AR gene displayed lower levels of serum androgens than women with shorter CAG repeats [21]. Although previous studies have demonstrated that the length of CAG repeats is not associated with risk of RA development, the effect of CAG repeats on onset age and rheumatoid positivity of RA patients does not have consistent results [22, 23]. Furthermore, all prior studies focus on the role of CAG repeats in RA, and no prior study has investigated GGC repeat polymorphism of the AR gene and RA. In light of the possible role of AR in RA disease activity and/or severity, our study aims to determine whether CAG or GGC length variations in the AR gene are correlated with the disease subtypes of RA in women.

Materials and methods

Patient and control populations This hospital-based case-control study investigates the relationship between CAG and GGC repeat lengths in the AR gene and female RA patients. A total of 111 female patients who fulfilled the 1987 revised American College of Rheumatology criteria for RA were recruited in this study [24]. The control population consisted of 104 healthy subjects who had no history of autoimmune disease, randomly selected in a 1:1 ratio, age of at least 18 years. Controls were also frequency matched to cases based on gender and 10-year age group. All individuals were Chinese residing in Taiwan.

We stratified RA patients based on a modification of the National Institute for Clinical Excellence/British Society for Rheumatology (NICE/BSR) guidelines [25, 26]. Our patients were all fit to two subtypes: refractory RA and remission RA. We excluded gray zone patients. Refractory RA patients comprised those who still had active disease despite 6 months of standardized combination therapy. Active disease denoted a disease activity score (DAS28)>5.1, measured at two points at least 3 months apart [27]. Failure of standardized combination therapy was defined as adequate therapeutic trials of nonsteroidal antiinflammatory drugs (NSAIDs), low-dose prednisolone (<10 mg/day, or equivalent dose), methotrexate (dose>15 mg per week for at least 3 months), and more than one of several other disease-modifying antirheumatic drugs (DMARDs; such as hydroxychloroquine, sulfasalazine, cyclosporine, penicillamine, azathioprine, IM gold or leflunomide) for at least 6 months. Patients with RA in stable remission must have fulfilled clinical remission criteria [28] and had only been treated with NSAIDs, low-dose prednisolone (<10 mg/day, or equivalent dose) with or without monotherapy for DMARDs for at least 6 months. The average DAS28 score in remission patients was less than 5.1.

Demographic and clinical features for participants were recorded on enrollment. Blood samples were taken at study entry and informed consent was obtained from all participants. Study was conducted according to protocol approved by the Chang-Gung Memorial Hospital ethics committee.

Molecular analysis of CAG and GGC repeat lengths Genomic DNA was extracted from whole blood samples frozen at −80°C using QIAamp DNA Mini kits (Qiagen, Hilden, Germany). To determine the number of CAG and

GGC repeats in the AR gene, polymerase chain reaction (PCR) products of CAG and GGC repeats were amplified using AmpliTaq GOLD Polymerase (Applied Biosystems, Foster City, CA, USA) with 5% dimethyl sulfoxide. Forward and reverse primer sequences of CAG and GGC repeats are as follows: CAG-F, 5′-ggtaagggaagtaggtggaa gattc-3′; CAG-R, 5′-cctctacgatgggcttggg-3′; GGC-F, 5′-cttcctcatcctggcacactc-3′; and GGC-R, 5′-ggcgacatttctggaag gaa-3′. Two forward primers labeled with 6-carboxyfluorescein were constructed. Cycling conditions were at an initial denaturation of 10 min at 95°C, followed by 30 cycles at 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 45 min, respectively. After PCR amplification, PCR products for CAG and GGC repeats from each sample were 50× diluted and added to 0.5 μl ROX Internal Size-Standard 500 (Applied Biosystems, Foster City, CA, USA) and 12 μl deionized formamide. The mixture was denatured at 95°C for 5 min and chilled on ice then run on an Applied Biosystems 3100 Genetic Analyzer using Pop-4 polymer. GeneScan software (Applied Biosystems, Foster City, CA USA) determined electrophoretic parameters. GeneScan data were analyzed with Genotyper software (Applied Biosystems, Foster City, CA, USA) to determine the number of CAG and GGC repeats in unknown samples. Resolution to one 1-bp length using this system was confirmed with direct DNA sequencing. CAG and GGC repeat lengths were defined based on the GeneBank sequence. We regenotyped 5% of our samples for quality control purposes.

Statistical analysis Comparisons of individual clinical and laboratory variables between RA subtypes and controls were assessed with one-way analysis of variance (ANOVA) for continuous data and the X^2 test for categorical data. Because the AR gene is mapped to chromosome X, men have one allele and women have two. Allele lengths are presented as mean ± standard deviation (SD). Differences in CAG and GGC repeat lengths between RA cases and control subjects were tested using Student's *t* test or one-way ANOVA test. CAG and GGC repeats were also assessed as categorical variables, based on both the mean and median of repeat numbers as the cut off and on previously published results [29, 30]. CAG repeat lengths of <23 were classified as short (S), and those ≥23 were classified as long (L). The GGC repeat lengths of <16 were classified as short (S), and those ≥16 were classified as long (L). Patients and control subjects were separated into subgroups comprising those with two short alleles (S/S), those with one short and one long allele (S/L), and those with both long alleles (L/L). Odds ratios (ORs) and 95% confidence intervals (CI) were calculated. We computed ORs using subjects homozygous for both short CAG and GGC alleles (S/S) as the reference. Statistical analysis utilized Statistical Package for the Social Sciences (SPSS) v11.5 for Windows (SPSS, Chicago, IL, USA). A two-tailed value of $p<0.05$ was considered statistically significant.

Results

Study population characteristics Complete data for the AR polymorphisms were collected for 111 RA subjects and 104 controls. Age of RA subjects and controls at recruitment did not differ significantly. Refractory RA patients were significantly younger at onset age than remission RA patients (41.0 vs 47.3 years; $p=0.009$). No significant difference was observed in rheumatoid factor (RF) positivity between RA subtypes. The erythrocyte sedimentation rate was higher in refractory RA than remission RA patients ($p<0.0001$; Table 1).

AR repeat characteristics Mean and median number of CAG repeats among refractory RA, remission RA cases and controls did not differ significantly (Table 2). Similarly, no significant differences existed between AR-gene GGC repeat lengths and RA disease subtypes in this study (Table 2). Two allele distributions were approximately normal for RA subtypes and controls and were similar to those obtained in other studies [29–32] (Fig. 1). The particular CAG repeats (such as 21, 22, 23) or GGC repeats (15, 16, 17, etc.) appeared to be not associated with RA (data not shown).

Compared to women who possessed the CAG S/S genotype, those who carried S/L (OR, 0.29; 95% CI, 0.09–0.90) had significantly increased risk of refractory RA than remission RA (Table 3). Similar results were observed for the risk of refractory RA in relation to having one or two L alleles [the S/L or L/L genotypes (OR, 0.3; 95% CI, [0.10–0.89]) when compared to women with the S/S genotype. In addition, we also compared the S/L or L/L genotypes of CAG repeats between refractory RA and control subjects. Any long allele in refractory RA had higher risk than controls (OR, 0.34; 95% CI, 0.12–0.96; data not shown); however, there is no significant difference between remission RA and controls. In contrast, no significant difference was found between GGC genotype and RA subtypes in this study (Table 3). Joint effects of CAG and GGC were not observed in relation to disease subtypes (data not shown).

AR repeats and allele zygosity status Because women have two AR gene alleles, homozygosity was defined as two repeats of the same length. Different lengths for two repeats indicated heterozygosity. The homozygous frequency of

Table 1 Comparison of demographic features between rheumatoid arthritis (RA) subtypes and controls

	RA subtypes		Control	p value
	Refractory RA	Remission RA		
Number of samples	51	60	104	
Age (years) at recruitment	50.6±11.0	55.5±11.1	52.3±15.8	0.151
Range	(22–75)	(28–76)	(20–78)	
Onset age (years)	41.0±12.2	47.3±12.5		0.009[a]
Range	(12–63)	(20–75)		
Onset≥55 years	6/51	20/60		0.007[b]
Onset<55 years	45/51	40/60		
Disease duration (years)	9.6±5.8	8.3±5.5		0.209
RF positivity (%)	42/51 (82.4%)	44/60 (73.3%)		0.257
ESR (mm/hr)	35.2±20.8	19.0±10.5	15.9±8.5	<0.0001[c]

$p<0.05$ indicated significance
RF, rheumatoid factor; *ESR*, erythrocyte sedimentation rate
[a] by t test; [b] by Chi-square test; [c] by one-way ANOVA test

two-allele CAG in refractory RA subjects was lower than that for remission RA subjects and controls ($p=0.042$; Chi-square test for the linear trends); this finding was not noted for the GGC allele ($p>0.05$; Table 2). The CAG and GGC homozygous frequency of control subjects in our study is similar to previous studies [33, 34].

AR repeats and RF positivity Subjects were considered seropositive if their RF result was ≥40 IU/ml and seronegative if their RF was <40 IU/ml (Reference value< 40 IU/ml; by nephelometry method). No differences in mean CAG and GGC lengths were observed between seropositive and seronegative RA patients (Table 4). Stratified by disease subtypes, mean CAG or GGC lengths in both groups were similar.

Discussion

Androgens constitute a class of steroid hormones that are essential for skeletal development as well as for the regulation of immune response throughout adult life. To direct immunoregulatory effects, AR functions as an androgen-inducible transcription factor to interact with cognate responsive DNA sequences of target genes and subsequently to activate the transcriptional activity of its target genes in various target cells [14, 15, 35, 36]. AR and its transcripts have been identified in synoviocytes, inflammatory cells, fibroblasts, and lining cells in RA knees, supporting the proposition that RA synovial tissue responds to androgens [37, 38]. Therefore, CAG and GGC repeat lengths in the AR gene that influence the activity of AR could be one of the important genetic factors in RA.

Table 2 Characteristics of CAG and GGC repeats between rheumatoid arthritis (RA) subtypes and controls

		RA subtypes		Control ($n=104$)	p value
		Refractory RA ($n=51$)	Remission RA ($n=60$)		
CAG repeat	Mean±SD	23.43±2.0	23.1±2.6	22.9±2.2	0.394[a]
	Median	23	23	23	
	Range	16–31	14–35	9–34	
	Heterozygosity %[c]	0.980	0.90	0.875	
	Homozygosity %[c]	0.02	0.10	0.125	0.042[b]
GGC repeat	Mean±SD	15.8±1.4	15.5±1.4	15.7±1.1	0.397[a]
	Median	16	16	16	
	Range	9–18	10–17	11–18	
	Heterozygosity %[c]	0.569	0.583	0.548	
	Homozygosity %[c]	0.431	0.417	0.452	0.758[b]

$p<0.05$ indicated significance
[a] by one-way ANOVA test; [b] by Chi-square test for the linear trend; [c] homozygosity was defined as two repeats of the same length, and different lengths for two repeats indicated heterozygosity.

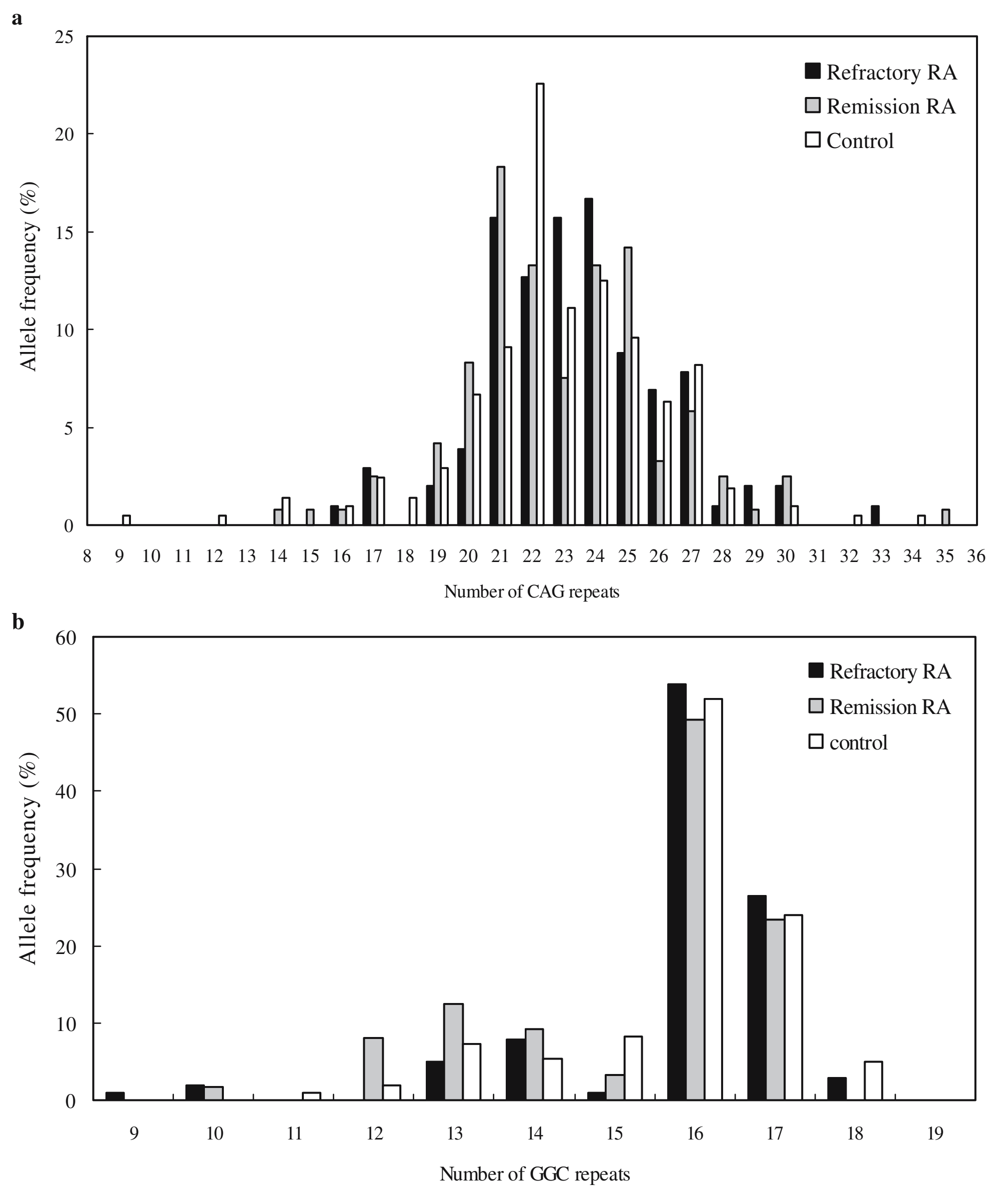

Fig. 1 **a** and **b** Frequency distributions of CAG and GGC repeat polymorphisms in androgen gene between RA subtypes and controls

Kawasaki et al. [22] have demonstrated that polymorphic CAG repeats in the AR gene was associated with the onset age of male but not female RA Japanese patients and androgen levels might be relevant to male RA patients only. However, the influence of AR-gene CAG repeat lengths on AR function in men and women may differ [21, 39]. A possible opposite role of CAG repeat lengths in carcinogenesis of male- and female-specific tumors has been suggested [31, 32, 40, 41]. Because controversy exists between CAG repeat lengths of AR polymorphism and RA in different races and gender, the question as to whether the AR gene determines an individual's susceptibility or the severity of RA need to be further clarified.

Previous studies have shown that the lengths of AR-gene GGC repeat lengths can also influence AR activity and are associated with several female-predominant diseases, including endometrial cancer, breast cancer, and esophageal cancer [42–44]. Although our results suggest that GGC repeat is not related to RA severity or activity, to our knowledge, our present study is the first report to investigate the association between AR-gene GGC repeats and RA subtypes in women.

Because RA represents a heterogeneous group of disorders with significant genetic components, linkage and association analyses have been widely applied to search for RA-susceptibility candidate genes and disease severity genes [1, 2]. Recent studies have suggested that HLA-DRB1*04 allele is the important predictor of disease severity in RA [1, 2]. The polymorphisms of tumor necrosis factor, interleukin (IL)-4, IL-1, and IL-1Ra may affect radiographic progression and outcomes [1, 2]. However, no strong evidence has linked AR polymorphism to different

Table 3 Association of CAG and GGC repeats with rheumatoid arthritis (RA) risk in different subtypes

	Refractory RA *n* (%)	Remission RA *n* (%)	OR (95% CI)	*p* value
CAG repeats				
S/S	5 (9.8)	16 (26.7)	1 (reference)	
S/L	29 (56.9)	27 (45)	0.29 (0.09–0.9)	0.028*
L/L	17 (33.3)	17 (28.3)	0.31 (0.09–1.05)	0.054
Any L	46 (90.2)	44 (73.3)	0.30 (0.10–0.89)	0.024*
GGC repeats				
S/S	6 (11.8)	13 (21.7)	1 (reference)	
S/L	5 (9.8)	7 (11.7)	0.65 (0.14–2.90)	0.567
L/L	40 (78.4)	40 (66.7)	0.46 (0.16–1.34)	0.148
Any L	45 (88.2)	47 (78.3)	0.48 (0.17–1.38)	0.167

OR, odds ratio; *CI*, confidence interval; CAG repeats: *S*, short <23 repeats; *L*, long ≥23 repeats; GGC repeats: *S*, short <16 repeats; *L*, long ≥16 repeats

*$p<0.05$ indicated significance

subtypes in RA. Our study observed a larger proportion of refractory RA patients carrying longer alleles with ≥23 CAG repeats than remission RA patients (OR, 0.30; 95% CI, 0.10–0.89; Table 3). It is likely that shorter CAG repeats seem to play a protective role against severe, refractory course of RA development. In contrast, we neither observed obvious combined effects of CAG and GGC repeats, nor the effect of GCC repeats alone between the RA subtypes.

In previous studies comparing Japanese and Caucasian populations, the homozygous frequency of CAG repeats was calculated to be 0.081–0.119 and the homozygosity rate of GGC repeats was 0.443–0.563 [33, 34]. Herein, we report for the first time, an investigation of the association between RA subtypes and CAG or GGC homozygosity. A trend was noted toward lower homozygous frequency of two-allele CAG in refractory RA, compared with remission RA or controls (Table 2). Although the biological mechanism by which homozygosity of CAG repeats acts in the RA phenotype is still not clear, mild clinical manifestations were observed in homozygous women with bulbospinal muscular atrophy (Kennedy disease) [45]. Our results may support this observation that homozygosity has greater possibility to enhance AR function or upregulate downstream AR targeted genes, then indirectly influence RA disease outcome. Because of limited sample size, our results may not have enough significance to strengthen the association between CAG/GGC repeats and disease status. Therefore, we are planning to increase the sample size of this study to further validate these results. Based on random X chromosome inactivation theory [46], one of the AR genes on two X chromosomes in women is activated, and the other should be inactivated. From our experimental study, the inability to distinguish between the active and inactive X allele of female RA subjects and controls was obviated by testing the risk differences between individuals with zero, one, or two alleles within a risk-category group (Table 3). Future methylation studies to measure allelic X-chromosome inactivation or AR function assays may be conducted to confirm the association between disease subtypes in RA and homozygosity of AR-gene CAG alleles.

Table 4 Comparison of mean CAG and GGC repeat lengths between rheumatoid factor (RF) positive and RF negative rheumatoid arthritis (RA) patients

		Refractory RA		Remission RA		*p* value[b]
		Number	Mean±SD	Number	Mean±SD	
CAG	RF positive	42	23.2±2.0	44	23.3±2.5	0.888
	RF negative	9	24.2±1.9	16	22.4±2.6	0.089
	p value[a]		0.20		0.222	
GGC	RF positive	42	15.9±1.2	44	15.4±1.5	0.077
	RF negative	9	15.3±2.1	16	15.8±1.1	0.457
	p value[a]		0.188		0.427	

$p<0.05$ indicated significance by *t* test

[a] *p* value difference between RF positive and RF negative groups; [b] *p* value difference between refractory RA and remission RA patients

Nevertheless, we also compared potential associations between AR-gene CAG/GGC repeats and onset age of RA. We found that CAG repeats were significantly shorter in the elderly-onset RA group (onset age ≥55 years) compared to the classic RA group (onset age <55 years; data not shown). These data suggest that AR gene polymorphisms may be involved in modulating genetic susceptibility to disease development between elderly-onset RA and classic RA. Further investigation is needed to determine whether shorter CAG repeat lengths in female RA patients are associated with delaying disease onset, or longer CAG repeat lengths in female RA patients may accelerate the development of RA.

In conclusion, this study demonstrated that the CAG repeat length is associated with RA subtypes. Possession of any long AR alleles (≥23 CAG repeats) by RA patients may be associated with increased risk of developing refractory subtype compared with women with two short *AR* alleles (<23 CAG repeats). The homozygosity of CAG but not GGC alleles is related to RA with clinical remission in Taiwanese women. Therefore, further prospective studies and more extensive studies involving large numbers of patients need to be performed. The development of preventive or therapeutic interventions is likely to depend upon the identification of genetic factors, such as AR, that underlie mechanisms that contribute to the potential role of AR microsatellite polymorphisms in RA progression.

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