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Full Length Research Paper

Association of HLA-G 14bp insertion/deletion polymorphism with autoantibody production in patients with autoimmune rheumatic diseases

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HLA-G is a non-classical HLA-class Ib molecule and has multiple immunoregulatory properties. A 14bp insertion/deletion polymorphism in the HLA-G gene has been suggested to influence the expression of HLA-G and then to associate with certain pathological conditions, including autoimmune diseases. The aim of study was to evaluate the possible association of the HLA-G 14bp insertion (+14bp) and deletion (-14bp) polymorphism with autoantibody production in patients with autoimmune rheumatic diseases. A total of 504 patients with rheumatic diseases and 367 unrelated healthy controls from a Chinese Han population were HLA-G genotyped for the 14bp insertion/deletion polymorphism. No statistically significant differences were observed in the frequencies of the HLA-G 14bp insertion/deletion alleles or genotypes between controls and patients with rheumatic disease. However, significant differences in the distribution of the HLA-G 14bp insertion/deletion polymorphism could be demonstrated for patients with positive anti-snRNP antibodies and patients with positive anti-histone antibodies when compared with the respective antibody-negative patients. The anti-snRNP antibodies positive group showed markedly increased frequencies of the +14bp allele (P = 0.0001, OR = 1.794, 95% CI = 1.329 - 2.422) as well as the +14/-14bp heterozygotes (P = 0.0001, OR = 2.306, 95% CI = 1.511 - 3.521). Moreover, an increased frequencies of the -14bp allele (P = 0.0001, OR = 2.302, 95% CI = 1.657 - 3.198) and the -14/-14bp homozygous genotype (P = 0.0001, OR = 3.035, 95% CI = 1.974 - 4.667) was observed in the patients with positive anti-histone antibodies. These findings are the first time to indicate that the HLA-G 14bp insertion/deletion polymorphism could be a genetic risk factor influencing the susceptibility for the autoantibody production in rheumatic diseases.

Key words: HLA-G, polymorphism, autoantibodies, rheumatic disease.

INTRODUCTION

HLA-G, a gene located within the major histocompatibility complex (MHC) at 6p21.3, has been referred to as a non-classical gene or class Ib because of its structural relation to classic MHC class Ia (HLA-A, HLA-B, HLA-C) (Apps et al., 2008). Studies have demonstrated a tolerogenic function for HLA-G molecule against innate and adaptive cellular immune responses (Rouas-Freiss et al.,

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1997). In normal tissues, HLA-G has only been detected on cytotrophoblast cells (Kovats et al., 1990), thymic epithelial cells (Crisa et al., 1997) and mature myeloid and plasmacytoid dendritic cells (Friec et al., 2004). However, the expression of HLA-G molecules has also been detected in pathological conditions, such as tumors (Wiendl et al., 2002; Gros et al., 2006), lymphoproliferative disorders (Sebti et al., 2003), allograft acceptance after transplantation (Lila et al., 2001) and inflammatory diseases (Wiendl et al., 2000; Fainardi et al., 2003). Moreover, the polymorphism in HLA-G gene regions has been showed to influence its molecule expression (Hviid et al., 2006; Chen et al., 2008). A 14bp sequence deletion/insertion polymorphism has been described in exon 8 in the 3'-untranslated region (UTR) of the HLA-G gene (rs16375) (Harrison et al., 1993). Many studies were conducted to evaluate the association of HLA-G gene 14bp deletion (-14bp) and insertion (+14bp) polymorphism with disease susceptibility. The association of HLA-G gene 14bp deletion/insertion polymorphism with disease susceptibility had been reported in idiopathic dilated cardiomyopathy (Lin et al., 2007), pre-eclamptic (Iversen et al., 2008), inflammatory diseases including systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (Rizzo et al., 2008).

The production of nonorgan-specific autoantibodies directed to nuclear, DNA, histone and small nuclear RNA proteins (snRNP), which may lead to a wide range of tissue injuries, is a hallmark of autoimmune diseases. Autoantibodies to histone are detected in more than 90% of the patients with drug-induced lupus and many kinds of other autoimmune diseases. Autoantibodies to snRNPs are found in -40% of SLE patients, in 100% of mixed connective tissue disease and at lower prevalence in other systemic rheumatic diseases (Tan, 1991). Though the precise mechanism of autoantibody production in auto- immune diseases is still unknown, both environmental and genetic factors are thought to be involved in the production of autoantibodies. Studies have shown complex genetic interactions that influence the production of autoantibodies (O'Hanlon et al., 2006; Schotte et al., 2004; Gottenberg et al., 2004; Sato et al., 2004; Gottenberg et al., 2003; Kuwana et al., 1999; Fanning et al., 1998; McHugh et al., 2006). It is possible that, genetic variants predisposing to the defective mechanisms of immunological tolerance, will allow antibody production against autoantigens. The purpose of this study was to investigate the question of whether the 14bp insertion/ deletion polymorphism in the HLA-G gene is associated with the production of autoantibodies, in a large cohort of 504 patients with immunological disorders.

MATERIALS AND METHODS

Patients and controls

A total of 504 patients with autoimmune diseases, including 231 patients satisfied the American College of Rheumatology (ACR) revised criteria for SLE (drug-induce SLE was excluded) (Hochberg, 1997), 154 patients met the ACR criteria for RA (Arnett et al., 1988), 31 patients met the European criteria for primary Sjögren's syndrome (pSS) (Vitali et al., 2002), 29 patients met the ACR criteria for systemic systemic sclerosis (SSc) (American Rheumatism Association, 1980), 22 patients met the criteria for mixed connective tissue disease (MCTD) (Kasukawa et al., 1987) and 37 patients fulfilled the criteria for undifferentiated connective tissue disease (UCTD) (Mosca et al., 2006), were consecutively recruited from five different Chinese Rheumatology centers. The mean age of patients was 35.7 years (range: 11 - 79 years) and median duration of diseases was 41 months, 87 of them were male patients and 417 were females. 367 unrelated healthy blood donors matched for sex and age from the same Chinese population served as controls. Written informed consent was obtained from all subjects and the study had approval from the ethical review committee of the five hospitals participating in the research.

HLA-G 14bp insertion/deletion evaluation

Peripheral blood mononuclear cells (PBMC) were obtained from EDTA-treated blood by Ficoll-Hypaque gradient centrifugation. Genomic DNA was extracted from the PBMC and stored at -20℃ till it is being genotyped. The HLA-G 14bp polymorphism in exon 8 (3'-UTR) of the HLA-G gene was identified by polymerase chain reaction (PCR) and performed as previously described [16].The PCR primers used were forward primer: 5`- GTG ATG GGC TGT TTA AAG TGT CAC C-3` and reverse primer: 5`-GGA AGG AAT GCA GTT CAG CAT GA-3`. Sample DNA (100 ng) was amplified in 25 µl of a reaction mixture containing 0.5 units of Taq DNA polymerase, dNTPs (2.5 µM each) (Takara Biotech. Co. Dalian, China), using an automated PCR thermal cycler (GeneAmp PCR System 2700; Applied Biosystems; Foster City, CA USA). Thermal cycling was performed with an initial 94℃ for 2 min followed by 30 cycles at 94℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ and a final extension at 72℃ for 5 min. The amplified products were visualized by electrophoresis on a 3% agarose gel (Oxoid Limited, Hampshire, England), containing ethidium bromide (0.5 mg/ml). PCR products were either 224 or 210bp, or both 224 and 210bp, depending on the insertion/deletion of the 14bp in exon 8. The number of 14bp insertion/deletion alleles was directly counted by three different observers (Figure 1).

Autoantibody measurement

Antinuclear antibodies (ANA) were measured by indirect immunofluorescence on Hep-2 cells. Antibodies to extractable nuclear antigens, including U1-snRNP, Sm, Ro/SSA (60 kDa and 52 kDa), La/SSB, histones, nucleosomes, Scl-70 and Jo-1, were tested by the immunoblot technique using a commercially procured kit (Euroimmun, Euroline: ANA profile 1). Anti-double stranded DNA (dsDNA) antibodies and anti-cyclic citrullinated peptide (CCP) antibodies were measured by enzyme linked immunosorbent assay (ELISA) (Euroimmun, Euroline).

Statistical analysis

All serological and genetic data were maintained on an Access database. Data were transferred to SSPS for statistical analysis. The HLA-G 14bp allele or genotype frequencies were tested for Hardy–Weinberg equilibrium. The difference of the HLA-G 14bp alleles or genotypes distribution between patients and healthy subjects was examined by Pearson Chi-Square test. The same test was also adopted for the study of the association of the various alleles and genotypes with the presence of certain autoantibody.

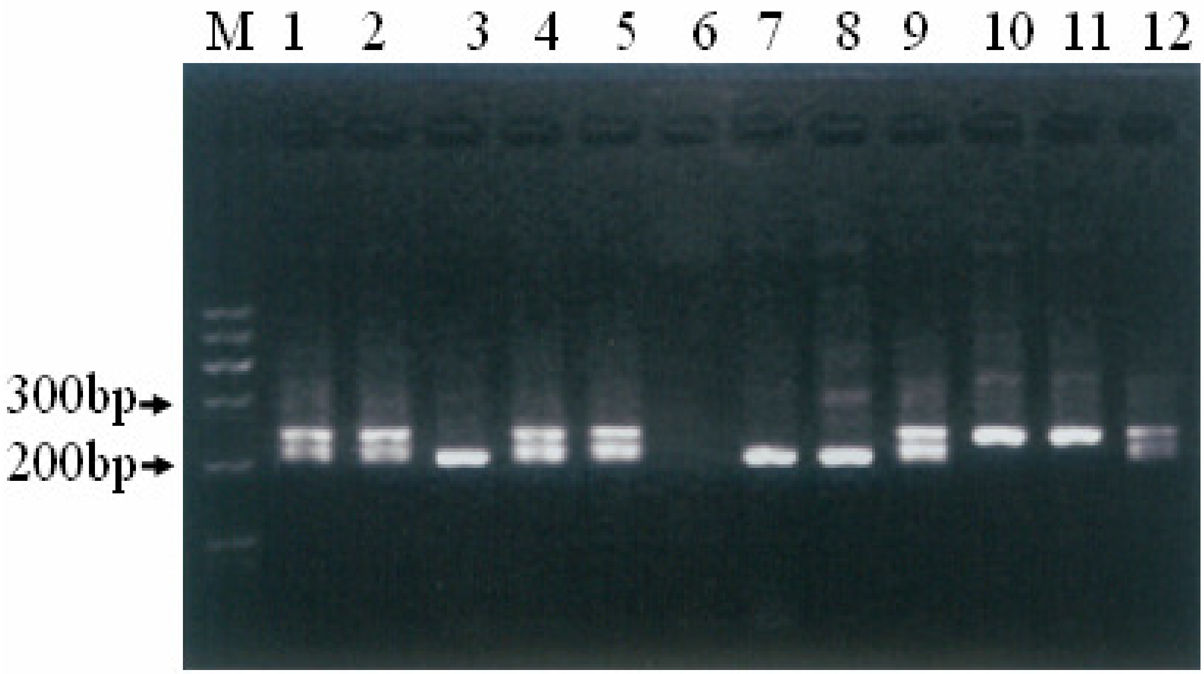

Figure 1. Representative agarose gel showing alleles of the HLA-G 14bp polymorphism. The 14bp insertion allele gave rise to a 214bp PCR product and the 14bp deletion allele to a 210-bp PCR product. Genotype assignments: heterozygous +14bp/-14bp, lanes 1, 2, 4, 5, 9, 12, homozygous -14/-14bp, lanes 3, 7, 8, homozygous +14/+14bp, lanes 10, 11, Negative control, lane 6, M, a DNA length marker.

The risk of autoantibody production due to the presence of an individual allele or genotype was calculated as the odds ratio (OR) and is given with the 95% confidence interval (CI). A P value of < 0.05 was taken as significant.

RESULTS

HLA-G 14bp insertion/deletion polymorphism in patients and controls

HLA-G 14bp insertion/deletion allelic frequencies were in Hardy–Weinberg equilibrium ($X^2 = 0.095$, P = 0.953 for the control group and $X^2 = 1.677$, P = 0.432 for the autoimmune rheumatic disease patients group). The frequencies of HLA-G 14bp insertion/deletion alleles and genotypes in patients with autoimmune rheumatic diseases and healthy subjects are reported in Table 1. No significant differences could be demonstrated between the SLE patients and healthy controls.

Association of HLA-G 14bp insertion/deletion polymorphism with autoantibody production

We further investigated whether the HLA-G 14bp insertion/deletion polymorphism had any influence on the autoantibody repertoire in patients with autoimmune diseases. The genotype distribution and allele frequencies of the HLA-G 14bp insertion/deletion polymorphism in patients with different autoantibody profiles are shown in Table 2. Using the χ^2 test, significant differences in the HLA-G 14bp insertion/deletion allele frequency and genotype distribution could be demonstrated for patients with positive anti-histone antibodies (P = 0.0001) and patients with positive anti-snRNP antibodies (P = 0.0001) compared with the respective antibody-negative patients (Table 2). In the patients with the presence of anti-histone antibodies, the HLA-G 14bp deletion (-14bp) allele was detected with a significantly increased frequency (75.4%, vs. the anti- histone antibodies negative group = 57.1%, $\chi^2 = 25.40$, P = 0.0001, OR = 2.302, 95% CI = 1.657 - 3.198). Meanwhile, an increased frequency of the HLA-G -14/-14bp homozygous genotype was observed in the anti-histone antibodies positive group compared with the anti-histone antibodies negative group (58.3, vs. 33.6%, χ^2=26.51, P = 0.0001, OR = 3.035, 95% CI = 1.974 - 4.667). Whereas, in the patients with positive anti-snRNP antibodies, the HLA-G 14bp insertion (+14bp) allele was detected with a significantly increased frequency (46.9%, vs the anti-snRNP antibodies negative group = 33.0%, χ^2 = 14.75, P = 0.0001, OR = 1.794, 95% CI=1.329 - 2.422). An increased frequency of the +14/-14bp heterozygotes was also observed in the anti-snRNP antibodies positive group compared with the anti- snRNP antibodies negative group (56.6 vs 36.1%, χ^2=15.29, P=0.0001, OR =2.306, 95% CI = 1.511 - 3.521). Odds ratios for the G allele in the subsets of patients with different autoantibodies are shown in Figure 2.

Table 1. HLA-G 14bp insertion/deletion allele frequencies and genotype distribution in healthy controls and patients with autoimmune rheumatic diseases.

	No. of subjects	HLA-G 14bp alleles				HLA-G 14bp genotypes				
		ins n (%)	del n (%)	X^2	P-value [a]	ins/ins n (%)	Ins/del n (%)	del/del n (%)	X^2	P-value [a]
HC	367	289 (39.4)	445 (60.6)			59 (16.1)	171 (46.6)	137 (37.3)		
SLE	231	177 (38.3)	285 (61.7)	0.134	0.714	40 (17.3)	97 (42.0)	94 (40.7)	1.217	0.544
RA	154	114 (37.0)	194 (63.0)	0.510	0.475	24 (15.6)	66 (42.9)	64 (41.5)	0.852	0.653
pSS	31	24 (37.7)	38 (62.3)	0.011	0.918	5 (16.1)	14 (45.2)	12 (38.7)	0.027	0.986
SSc	29	21 (36.2)	37 (63.8)	0.226	0.634	4 (13.8)	13 (44.8)	12 (41.4)	0.223	0.894
MCTD	22	20 (45.5)	24 (54.5)	0.641	0.423	4 (18.2)	12 (54.5)	6 (27.3)	0.907	0.635
UCTD	37	31 (41.9)	43 (58.1)	0.178	0.673	7 (18.9)	17 (45.9)	13 (35.2)	0.213	0.899

Abbreviations: ins = 14bp insertion, del = 14bp deletion, ins/ins = homozygous +14bp, ins/del = heterozygous, del/del = homozygous -14bp, HC = healthy controls, SLE = systemic lupus erythematosus, RA = rheumatoid arthritis, pSS= primary Sjögren's syndrome, SSc= systemic systemic sclerosis, MCTD = connective tissue disease, UCTD = undifferentiated connective tissue disease. [a] P-values were calculated using the healthy controls as reference.

DISCUSSION

An ample amount of evidence demonstrates that HLA-G plays a vital role in immune modulation. It is involved in the modulation of the cytotoxicity of natural killer cells and CD8+ T-cell, inhibiting T-cell proliferation and dendritic cell maturation, through direct binding to the inhibitory receptors ILT-2 (LILRB1/ CD85j), ILT-4 (LILRB2/ CD85d) and KIR2DL4 (CD158d) (Riteau et al., 2001; LeMaoult et al., 2004; Kanai et al., 2001). Recent studies suggest a role of HLA-G in the pathogenesis of autoimmune diseases. The altered expression of HLA-G had been documented in many kinds of autoimmune diseases, including ulcerative colitis, multiple sclerosis, rheumatoid arthritis, inflammatory myopathies and SLE (Wiendl et al., 2000; Torres et al., 2004; Wiendl et al., 2005; Verbruggen et al., 2006; Wiendl et al., 2003; Rosado et al., 2008); suggesting that differential expression of the HLA-G molecule might play a role on the immunopathology of autoimmune diseases. A 14bp insertion/deletion polymorphism in exon 8 in the 3'-untranslated region (UTR) of the HLA-G gene (rs16375) has recently gained its interest. The association of this polymorphism with certain pathological conditions, such as idiopathic dilated cardiomyopathy, pemphigus vulgaris and pathological pregnancies (Lin et al., 2007; Iversen et al., 2008), has been addressed. In autoimmune disorders, Veit et al. (2008) reported that female patients with juvenile idiopathic arthritis presented a higher frequency of the 14bp deletion allele when compared with female healthy children. Rizzo et al. (2008) had genotyped the HLA-G 14bp insertion/ deletion polymorphism in 200 Italian SLE patients and found a significant increased frequency of the +14/+14bp homozygote paralleled with a decreased frequency of the -14/-14bp genotype in SLE patients.

In this study, we had the HLA-G 14bp deletion/ insertion polymorphism genotyped in 231 patients with SLE, and 154 patients with RA. No association of the HLA-G 14bp insertion/deletion polymorphism with SLE or RA had been found. Our results are in line with those of Rizzo et al. (2006) and Veit et al. (2009) in the sense that the insertion allele is not a risk factor for RA or SLE. Due to the relatively small number of patients enrolled in our study, the HLA-G 14bp insertion/ deletion polymerphism as a genetic factor influencing the susceptibility of other rheumatic diseases, including pSS, SSc, MCTD and UCTD, could not be clarified yet.

The hallmark of the disease process in autoimmune disorders is the production of a large array of autoantibodies, including autoantibodies directed to nuclear (ANA), single- and double-stranded DNA, histone, some nuclear RNA proteins (Sm, nRNP) and cytoplasmic antigens (Ro, La). We therefore, investigated whether the HLA-G 14bp insertion/deletion polymorphism had any influence on the autoantibody repertoire in patients with autoimmune diseases. We found a highly significant association of the HLA-G 14bp insertion allele as well as the +14/-14pb heterozygote with the presence of anti-snRNP antibodies. Whereas, the 14bp deletion allele and the -14/-14bp homo- zygote were found to be significantly associated with the production of anti-histone antibodies.

Anti-snRNP antibodies and anti-histone antibodies are common auto-antibodies found in

Table 2. Association of the HLA-G 14bp insertion/deletion polymorphism with autoantibody production.

Auto-Abs		No. tested n (%)	HLA-G 14bp alleles			HLA-G 14bp genotypes			
			ins n (%)	del n (%)	P-value	ins/ins n (%)	ins/del n (%)	del/del n (%)	P-value
ANA	Present	349 (83.7)	263 (37.7)	435 (62.3)	0.599	58 (16.6)	147 (42.1)	144 (41.3)	0.785
	Absent	68 (16.3)	48 (35.3)	88 (64.7)		9 (13.2)	30 (44.1)	29 (42.6)	
Anti-dsDNA	Present	113 (27.1)	95 (42.0)	131 (58.0)	0.084	23 (20.3)	49 (43.4)	41 (36.3)	0.245
	Absent	304 (72.9)	216 (35.5)	392 (64.4)		44 (14.5)	128 (42.1)	132 (43.4)	
Anti-Sm	Present	71 (17.0)	56 (39.4)	86 (60.6)	0.561	14 (19.7)	28 (39.4)	29 (40.9)	0.637
	Absent	346 (83.0)	255 (36.9)	437 (63.1)		53 (15.3)	149 (43.1)	144 (41.6)	
Anti-histone	Present	128 (31.7)	63 (24.6)	193 (75.4)	0.0001	12 (12.2)	39 (29.5)	77 (58.3)	0.0001
	Absent	289 (69.3)	248 (42.8)	330 (57.1)		55 (19.2)	138 (48.3)	96 (33.6)	
Anti-SSA(Ro)	Present	158 (37.9)	125 (39.6)	191 (60.1)	0.290	31 (19.6)	63 (39.9)	64 (40.5)	0.293
	Absent	259 (62.1)	186 (35.9)	332 (64.1)		36 (13.9)	114 (44.0)	109 (42.1)	
Anti-SSB(La)	Present	69 (16.5)	58 (42.0)	80 (58.0)	0.208	10 (14.5)	38 (55.1)	21 (30.4)	0.059
	Absent	348 (83.5)	253 (36.4)	443 (63.6)		57 (16.4)	139 (39.9)	152 (43.7)	
Anti-snRNP	Present	129 (30.9)	121 (46.9)	137 (54.1)	0.0001	24 (18.6)	73 (56.6)	32 (24.8)	0.0001
	Absent	288 (69.1)	190 (33.0)	386 (67.0)		43 (14.9)	104 (36.1)	141 (49.1)	
RF	Present	201 (64.0)	150 (37.3)	252 (62.7)	0.941	33 (16.4)	84 (41.8)	84 (41.8)	0.843
	Absent	113 (36.0)	85 (37.6)	141 (62.4)		17 (15.0)	51 (45.1)	45 (39.8)	
CCP	Present	147 (46.8)	106 (36.1)	188 (63.9)	0.964	24 (16.3)	58 (39.5)	65 (44.2)	0.458
	Absent	167 (53.2)	121 (36.2)	213 (63.8)		22 (13.2)	77 (46.1)	68 (40.7)	

Abbreviations: ins = 14bp insertion, del = 14bp deletion, ins/ins = homozygous +14bp, ins/del = heterozygous, del/del = homozygous -14bp, ANA = anti-nuclear antibodies, RF=rheumatoid factor, CCP= cyclic citrullinated peptide, P - values corrected for multiple comparisons were calculated

autoimmune rheumatic diseases, including SLE, RA, pSS, SSc, MCTD and UCTD. The molecular mechanism underlying the production of anti-snRNP or anti-histone antibodies is unknown, but genetic factors are probably implicated in the pathogenesis. The loss of immune tolerance to self-components is the basis of auto-antibody production, so many genes encoding proteins with regulatory or adaptive functions in the immune system have been considered as candidates predisposing to development of auto-antibodies. Since the HLA molecules present processed antigen to the TCR and result in an antigen-specific immune response, studies have been conducted to clarify the possible associations between HLA antigens and particular antibodies. The production of snRNP antibodies has been associated with HLA-DR4 [44] or HLA-DR7 (Rouas-Freiss et al., 1997) in Caucasian and black patients with SLE, and with DR2 or DR4 in Japanese patients with MCTD (Kuwana et al., 1996; Kaneoka et al.,1992). Analyzing black and white patients with either anti-Sm or RNP precipitin autoantibodies, Olsen et al. (1993) have shown that there are distinct patterns of major HLA class II allele associations according to the racial origin of patients and the specificity of antibodies. Also, it was found by others that the frequency of HLA-DR3 was increased among patients with drug-induced SLE which was dominated by the presence of anti-histone antibodies (Gunnarsson et al., 1997). Be noted, strong linkage disequilibrium between particular HLA-DR, HLA-DQ alleles and HLA-G was also observed (Ober et al., 1996; Hviid and Christiansen, 2005). Thus, it could be expected that either HLA-G alone or together with linkage disequilibrium alleles could play roles in the development of auto-antibodies. More work is needed to elucidate the mechanisms underlying the association of the HLA-G 14bp insertion/deletion polymorphism with antibody production in autoimmune rheumatic diseases.

In summary, our data demonstrate that the HLA-G 14bp insertion/deletion polymorphism does not contribute significantly to susceptibility to autoimmune rheumatic

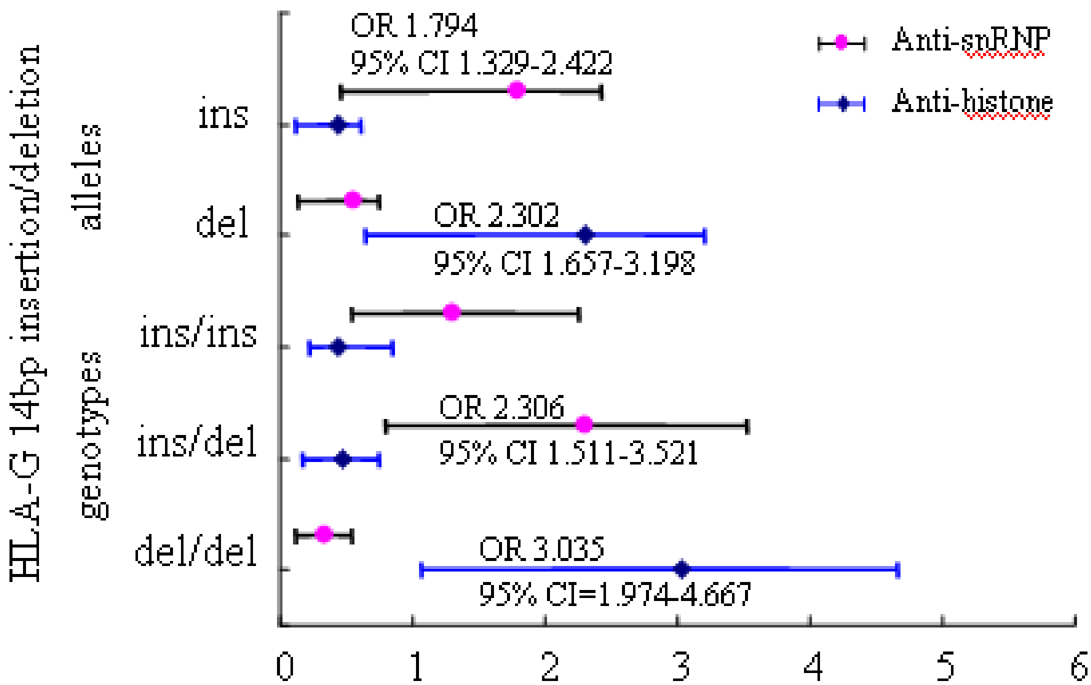

Figure 2. OR and 95% CI for the HLA-G 14bp insertion/deletion alleles and genotypes in patients with anti-snRNP and anti-histone autoantibodies.

diseases, but predisposes to the production of anti-snRNP and anti-histone antibodies. More work is needed to elucidate the mechanisms underlying the association of the HLA-G 14bp insertion/deletion polymorphism with antibody production in autoimmune rheumatic diseases.

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