Association between CTLA-4 polymorphisms and the susceptibility to systemic lupus erythematosus and Graves'disease in Thai population

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Summary

Background: **Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a cell surface molecule involved in the regulation of T cells. Single nucleotide polymorphisms (SNPs) of CTLA-4 gene are known to be associated with susceptibility to several autoimmune diseases, including systemic lupus erythematosus (SLE) and Graves' disease (GD).**

Objective: **The aim of this study was to determine whether the common SNPs +49A/G on exon1 and CT60A/G in 3'UTR of the CTLA-4 gene are associated with susceptibility to SLE and GD in Thai population.**

Methods: **Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to analyze these two SNPs in 151 patients with SLE, 132 patients with GD and 153 healthy controls.**

Results: **Our study showed that there were no statistically significant differences in the allele and genotype frequencies of +49A/G and CT60A/G SNPs between patients with SLE and healthy controls as well as patients with GD vs. healthy controls (*P* >0.05). However, the GG genotypes of +49A/G and CT60A/G were likely to be risk factors (OR >1) for GD but not in SLE. The effect of the +49G allele was similar to that of an autosomal recessive gene in the presence of the GG genotype, when compared to AA and AG, with an OR of 1.58 (95% CI =0.95-2.61, *p* =0.061) in GD. We also observed a dose response effect of the CT60G allele on GD susceptibility with an OR of 1.43 for GG homozygous and 1.17 for AG heterozygous subjects, when compared to the AA genotype, although these were not statistically significant (*P* >0.05).**

Conclusion: **We found no association between two functional polymorphisms (+49A/G and CT60A/G) of the CTLA-4 gene and susceptibility to SLE and GD. However, the association study utilizing a larger sample size should be performed to confirm this.** ***(Asian Pac J Allergy Immunol 2011;29:229-35)***

Key words: *Systemic lupus erythematosus; Graves' disease; CTLA-4; polymorphisms*

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Introduction

Systemic lupus erythematosus (SLE) and Graves' disease (GD) are autoimmune disorders in which the body produces autoantibodies against self-antigens. The characteristics of SLE include the production of autoantibodies directed at nuclear, cytoplasmic and cell surface autoantigens. These autoantibodies cause end-organ damage via an inflammatory response to immune complexes. In GD the body produces autoantibodies to thyroid stimulating hormone receptors, leading to hyperthyroidism. Although the etiopathogenesis remains elusive, genetic factors seem to be important

Table 1. Clinical manifestations in 119 patients with SLE

Clinical manifestation	Number of patients with SLE (%)
Malar rash	84 (70.59)
Discoid rash	48 (40.34)
Photosensitivity	50 (42.02)
Oral or Nasal Ulcers	52 (43.70)
Arthritis	93 (78.15)
Pleurisy or Pericarditis	6 (5.04)
Renal Disorder	73 (61.34)
Neurologic Disorder	9 (7.56)
Hematologic Disorder	87 (73.11)
Immunologic Disorder	48 (40.34)
Positive Anti-nuclear antibody	108 (90.76)

in the development of these two diseases. Twins studies show significantly higher concordance rates in monozygotic than in dizygotic twins (more than 10 times greater).[1,2]

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a member of the immunoglobulin superfamily, which is expressed on the surface of activated T cells. It shares sequence homology with T cell co-stimulatory protein CD28. Both molecules bind to the same ligands, B7.1 (CD80) and B7.2 (CD86), but have opposing functions. CD28 promotes a number of T-cell activities, whereas CTLA-4 is a negative regulator of T cell responses.[3] Several studies have reported an association between CTLA-4 gene polymorphisms and several different autoimmune diseases including SLE and GD.[4] There have been several single nucleotide polymorphisms identified (SNPs), which are associated with these two diseases, such as CT60A/G in 3'-untranstrated region (3'UTR), +49A/G on exon 1, -1772T/C, -138C/T in promoter region and 106 bp allele of a dinucleotide repeat in 3' UTR.[5-23]

However, those association studies have produced conflicting results. In addition, the susceptibility variants in the CTLA-4 gene might differ among the different ethnic groups. Thus, we performed an association study examining the role of CTLA-4 SNPs in Thai patients. The functional SNPs are believed to be mainly in +49A/G (rs231775) and CT60A/G (rs3087243). The +49A/G SNP causes an amino acid change from threonine to alanine in the peptide leader sequence of the CTLA-4 protein,[24] whereas CT60A/G is important for efficient splicing and production of soluble CTLA-4, and may plays role in mRNA stability of sCTLA-4.[25]

Therefore, the aim of this study was to investigate the association between these two common SNPs (+49A/G and CT60A/G) within the CTLA-4 gene and the susceptibility to SLE and GD in Thai population.

Methods

Subjects

One hundred and fifty-one Thai patients (148 women and 3 men: mean age ± SD = 36.21 ± 10.76 years) from King Chulalongkorn Memorial hospital, who fulfilled at least 4 of the American College of Rheumatology (ACR) revised criteria for SLE were included in this study.[26] Clinical and serological data were recorded as either absent or present, based on the data from the cumulative database obtained by chart review. Complete clinical data were obtained from only 119 patients with SLE as shown in Table 1. In addition, we recruited 132 patients with GD (116 women and 16 men: mean age ± SD = 38.27 ± 12.41 years) followed up in an outpatient clinic at the Department of Endocrinology, Faculty of Medicine, King Chulalongkorn Memorial Hospital (Bangkok, Thailand). The diagnosis of GD was based on clinical features, diffuse enlargement of thyroid gland and elevation of free thyroxine or triiodothyronine levels for more than 3 months with positive thyroid autoantibodies (TBII or TPO), thyroid eye disease or a diffuse increase in uptake on radionuclide scanning. For the control group, we recruited 153 ethnically matched healthy donors from the Thai Red Cross Society (97 women and 56 men: mean age ± SD = 23 ± 12.3 years). The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent.

DNA extraction and genotyping study

DNA was extracted from the buffy coat, collected with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, using a salting-out method.[27] DNA was aliquoted and stored at 20 °C until used. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to analyze the polymorphism of CTLA-4 at exon1 position +49A/G[28] and CT 60A/G in 3'UTR as previously described.[10] Negative controls without a DNA template were included in each experiment. Ten percent of the samples were confirmed by direct sequencing of PCR products to verify the accuracy of genotyping.

Table 2. Genotype and allele frequencies of *CTLA4* gene polymorphisms in SLE, GD patients and control subjects

SNP	Genotype /allele	SLE	GD	Healthy control	SLE vs. Healthy control		GD vs. Healthy control	
		N = 151 (%)	N = 132 (%)	N = 153 (%)	OR (95% CI)	*P* value	OR (95% CI)	*P* value
+49 A/G	AA	33 (21.85)	22 (16.67)	26 (16.99)	1.00		1.00	
(rs231775)	AG	77 (50.99)	49 (37.12)	73 (47.71)	0.83 (0.43-1.59)	0.549	0.79 (0.38-1.64)	0.500
	GG	41 (27.15)	61 (46.21)	54 (35.29)	0.60 (0.29-1.21)	0.123	1.34 (0.64-2.77) [a]	0.401
	A	143 (47.35)	93 (35.23)	125 (40.85)	1.00		1.00	
	G	159 (52.65)	171 (64.77)	181 (59.15)	0.77 (0.55-1.07)	0.106	1.27 (0.89-1.81)	0.168
CT60 A/G	AA	10 (6.62)	8 (6.06)	12 (7.84)	1.00		1.00	
(rs3087243)	AG	74 (49.01)	46 (34.85)	59 (38.56)	1.51 (0.56-4.08)	0.374	1.17 (0.4-3.45)	0.753
	GG	67 (44.37)	78 (59.09)	82 (53.59)	0.98 (0.37-2.63)	0.966	1.43 (0.51-4.07)	0.460
	A	94 (31.13)	62 (23.48)	83 (27.12)	1.00		1.00	
	G	208 (68.87)	202 (76.52)	223 (72.88)	0.82 (0.57-1.19)	0.277	1.21 (0.81-1.81)	0.320

SLE, Systemic lupus erythematosus; GD, Graves' disease; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval

[a] autosomal recessive model GG/AA+AG; OR (95% CI)= 1.58 (0.95-2.61), p =0.061

Statistical Analysis

Genotype frequencies were checked for consistency among normal controls with those expected from the Hardy–Weinberg equilibrium (HWE). Allele and genotype frequencies were compared between groups using the chi-squared (χ^2) test or Fisher's exact probability test, where appropriate. The PLINK v1.07 program was used to calculate HWE, *P* -values, odds ratios and 95% confidence intervals, as well as for linkage disequilibrium (LD) and haplotype analysis.[29] A *P*-value of <0.05 was considered statistically significant. In addition, the sample size and the power for our genetic association study were calculated using the PS program.[30] The sample size calculation was based on the ratio of control to case patients (1:1), probability of exposure among controls (0.310[6] for SLE; 0.383[19] for GD in SNP +49A/G and 0.466[10] for SLE; 0.781[19] for GD in SNP CT60A/G) and an odds ratio of 2.11[6], 1.75[19], 1.71[10] and 1.42[19], respectively. By this calculation, the minimum sample sizes to study are 130 SLE vs. 130 controls; 218 GD vs. 218 controls for SNP +49A/G and 236 SLE vs. 236 controls; 868 GD vs. 868 controls for SNP CT60A/G to be able to reject the null hypothesis that this odds ratio equals 1 with probability (power) 0.8.[30]

Results

The distribution of genotype and allele frequencies of CTLA-4 polymorphisms in patients with SLE, GD and control subjects is shown in Table 2. In the present study, both SNPs were in Hardy–Weinberg equilibrium when compared with the observed and expected genotype frequencies of each SNP (*P* >0.05). There were no statistically significant differences in the allele and genotype frequencies of +49A/G and CT60A/G SNPs within CTLA-4 between patients with SLE and healthy controls, as well as patients with GD vs. healthy controls (*P* >0.05). However, the GG genotype of +49A/G and CT60A/G was likely to be a risk factor (OR >1) in GD, although this was not statistically significant (*P* >0.05). The effect of +49G allele was similar to an autosomal recessive gene, in which the presence of GG genotype when compared to AA and AG with an OR of 1.58 (95% CI =0.95-2.61, p =0.061). Furthermore, we found a dose response effect of the CT60G allele on GD susceptibility with an OR of 1.43 for GG homozygous and 1.17 for AG heterozygous, when compared to the AA genotype (Table 2.). The association of CTLA-4 gene polymorphisms with clinical manifestations of SLE was also analyzed. In this study, there was no significant association between CTLA-4 polymorphism with any clinical manifestations in SLE patients (data not shown).

Table 3. Association studies between the CTLA-4 polymorphisms and the susceptibility to SLE and GD in Thai and other Asian populations

Study	Population	Sample size		Finding	Reference
		SLE	Control		
Exon 1+49A/G					
Matsushita M et al., 1999	Japanese	71	150	non-significant	31
Liu MF et al., 2001	Chinese	81	81	non-significant	32
Lee YH et al., 2001	Korean	80	86	non-significant	33
Ahmed S et al., 2001	Japanese	113	200	OR for allele G = 1.72 (*P*=0.003); OR for GG vs. AG+AA = 2.11 (*P*=0.003)	6
Hudson LL et al., 2002	Korean	130	200	non-significant	7
This study	Thai	151	153	non-significant	
3'UTR CT60A/G					
This study	Thai	151	153	non-significant	
Study	**Population**	**Sample size**		**Finding**	**Reference**
		GD	**Control**		
Exon 1+49A/G					
Yanagawa T et al., 1997	Japanese	153	200	least one G allele (GG or AG) ; *P*<0.01	11
Han SZ et al., 2006	Chinese	263	196	OR for allele G = 1.45 (*P*=0.008); OR for GG vs. AG+AA = 1.56 (*P*=0.02)	19
Zhao SX et al., 2010	Chinese	2640	2204	OR for allele G = 1.24 (*P* = 9.39x10-5)	23
This study	Thai	132	153	non-significant	
3'UTR CT60A/G					
Ban Y et al., 2005	Japanese	131	179	OR for allele G = 2.00 (*P*=0.0008); OR for GG vs. AG+AA = 2.00 (*P*=0.004)	16
Weng YC et al., 2005	Taiwanese	107	101	OR for allele G = 1.89 (*P*=0.011); OR for GG vs. AG+AA = 2.34 (*P*=0.004)	17
Han SZ et al., 2006	Chinese	263	196	OR for allele G = 1.42 (*P*=0.04)	19
Cho HJ et al., 2006	Korean	278	472	non-significant	34
Tsai ST et al., 2008	Taiwanese	189	620	OR for allele G = 1.71 (*P*=0.006); OR for GG vs. AG+AA = 1.61 (*P*=0.0049)	21
This study	Thai	132	153	non-significant	

SLE, Systemic lupus erythematosus; GD, Graves' disease; OR, odds ratio

In addition, we compared our study to the association studies in other Asian populations (Table 3.). Our study of SNP +49A/G showed no significant association with SLE, which was similar to the findings of other studies in Japanese,[31] Chinese,[32] and Korean[7,33] patients, with the exception of the study reported by Ahmed S et al. [6] They found that Japanese SLE patients had a significantly higher frequency of the +49G allele (OR =1.72, *P* =0.003) and GG genotype (OR=2.11, *P* =0.003) than controls.[6] For +49A/G in GD, we also found no significant association, which was inconsistent with the studies in Japanese[11] and Chinese[19,23] patients. These studies found that the frequency of the +49G allele and GG genotype was significantly higher in GD than controls (*P* <0.05). In terms of SNP CT60A/G, there were no studies in Asian SLE patients available for comparison. However, four previous reports[16,17,19,21] showed results consistent with those of our study and a study by Cho HJ et al[34], that is that the G allele and GG genotype were important in GD development (*P* <0.05).

Table 4. Haplotype analysis for +49 A/G (rs231775) and CT60 A/G (rs3087243)

Haplotype (MHF≥0.05)	Haplotype frequency			SLE vs. Healthy control		GD vs. Healthy control	
	SLE	GD	Healthy control	OR (95%CI)	*P* value	OR (95%CI)	*P* value
AA	0.3020	0.2319	0.2712	1.15 (0.80 - 1.65)	0.4030	0.81 (0.56 - 1.18)	0.2823
AG	0.1779	0.1217	0.1373	1.35 (0.86 - 2.12)	0.1706	0.87 (0.53 - 1.42)	0.5817
GG	0.5201	0.6464	0.5915	0.72 (0.52 - 1.00)	0.0776	1.23 (0.89 - 1.70)	0.1794

MHF, Minor haplotype frequency; SLE, Systemic lupus erythematosus; GD, Graves' disease; OR, odds ratio; CI, confidence interval

Moreover, we performed haplotype analysis of these two SNPs with a strong LD (D' =0.952). There were three common haplotypes (minor haplotype frequency ≥ 0.05) including AA, AG and GG. To test the association of the CTLA-4 haplotype and disease development, we compared each tested haplotype with two other haplotypes, between patients and controls. In this study, we did not find any significant association between CTLA-4 haplotypes and either disease (Table 4.).

Discussion

In the present study, we determined the association between the CTLA-4 polymorphisms and the susceptibility to SLE and GD. We focused on two functional polymorphisms, comprising +49A/G SNP on exon 1(rs231775) and CT60A/G SNP in 3'UTR (rs3087243). Our results showed no significant association of +49A/G and CT60A/G polymorphisms with SLE. These results confirm six previous reports in which no association of +49A/G SNP with SLE,[7,9, 28, 31-33] was found, although two other studies have found evidence of an association.[5,6] These studies reported that the frequency of the +49G allele and GG genotype was significantly higher in SLE patients than in controls.[5,6] This +49G allele was associated with decreased control of T lymphocyte proliferation.[35,36] In addition, individuals carrying the GG genotype had reduced cell surface expression of CTLA-4 when compared to the AA genotype. [37] In contrast to the study by Ulker M et al (2009), they showed a relationship between the AA genotype and development of SLE in Turkish patients.[38] These conflicting results might be because of different populations, although the results of Matsushita M et al[31] differed from those of Ahmed S et al[6], which involved the same population (Japanese). This may be because the study by of Matsushita M et al study is underpowered (sample size 71 SLE patients and 150 controls), whereas Ahmed S et al had 113 SLE patients vs. 200 controls. We calculated the power based on their sample sizes, probability of exposure among controls (0.310) and the OR of 2.11[6] using the PS program[30]. This calculation showed that the study of Matsushita M et al[31] had a lower power (66.6%) than Ahmed S et al[6] study (84.5%). With respect to the CT60A/G SNP, a study showed an association for this SNP in Spanish patients with SLE. In that study, they found the frequency of the CT60G allele was significantly increased among SLE patients compared with control subjects.[10] However, this was not replicated in our Thai patients with SLE.

For our study in GD, there were no statistically significant differences in the allele and genotype frequencies of +49A/G and CT60A/G SNPs within CTLA-4 gene between patients with GD and healthy controls. However, the GG genotype of these two SNPs was likely to be a risk factor for disease development, although this was not statistically significant. This might be due to the limited sample size. The power for our genetic association study was calculated using the PS program,[30] based on our sample size (132 cases vs. 153 controls), probability of exposure among controls (0.383 for +49A/G and 0.781 for CT60A/G)[19] and an odds ratio of 1.75[19] and 1.42.[19] From this calculation, our study had a power of 60.0% for +49A/G and 16.6% for CT60A/G, with $\alpha = 0.05$. To improve the power of detection, the sample sizes should be increased in further studies.

The CT60G allele has been shown to influence the efficiency of splicing and lower production of soluble CTLA-4 (sCTLA-4).[25] The sCTLA-4 has a B7.1 (CD80) and B7.2 (CD86) recognition site. The binding of sCTLA-4 to CD80/86 molecules inhibits T-cell proliferation *in vitro*.[39] Thus, the reduction of sCTLA-4 could lead to decreased impeding of CD80/CD86, resulting in an increasing of T cell activation. An association of the CT60G allele with GD was reported in seven studies, although it was not associated with GD in the study of Cho HJ et

al.[14-19,21,34] A meta-analysis suggests strong evidence that the CT60 GG genotype has a greater risk of developing GD than GA.[40] We found a dose response effect of the CT60G allele on GD susceptibility, but there was no statistically significant relationship between them. The GG homozygous (OR = 1.43) has a greater tendency to cause GD than AG heterozygous (OR = 1.17), when compared to the AA genotype. This result indicates that two G alleles are required to exhibit a risk effect, whereas one G allele might not be sufficient. An association study utilizing a larger sample size should be performed to further verify.

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References

1. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, et al. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum. 1992 Mar;35:311-8.
2. Brix TH, Kyvik KO, Christensen K, Hegedus L. Evidence for a major role of heredity in Graves' disease: a population-based study of two Danish twin cohorts. J Clin Endocrinol Metab. 2001 Feb;86:930-4.
3. Teft WA, Kirchhof MG, Madrenas J. A molecular perspective of CTLA-4 function. Annu Rev Immunol. 2006;24:65-97.
4. Gough SC, Walker LS, Sansom DM. CTLA4 gene polymorphism and autoimmunity. Immunol Rev. 2005 Apr;204:102-15.
5. Pullmann R, Jr., Lukac J, Skerenova M, Rovensky J, Hybenova J, Melus V, et al. Cytotoxic T lymphocyte antigen 4 (CTLA-4) dimorphism in patients with systemic lupus erythematosus. Clin Exp Rheumatol. 1999 Nov-Dec;17:725-9.
6. Ahmed S, Ihara K, Kanemitsu S, Nakashima H, Otsuka T, Tsuzaka K, et al. Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. Rheumatology (Oxford). 2001 Jun;40:662-7.
7. Hudson LL, Rocca K, Song YW, Pandey JP. CTLA-4 gene polymorphisms in systemic lupus erythematosus: a highly significant association with a determinant in the promoter region. Hum Genet. 2002 Oct;111:452-5.
8. Fernandez-Blanco L, Perez-Pampin E, Gomez-Reino JJ, Gonzalez A. A CTLA-4 polymorphism associated with susceptibility to systemic lupus erythematosus. Arthritis Rheum. 2004 Jan;50:328-9.
9. Barreto M, Santos E, Ferreira R, Fesel C, Fontes MF, Pereira C, et al. Evidence for CTLA4 as a susceptibility gene for systemic lupus erythematosus. Eur J Hum Genet. 2004 Aug;12:620-6.
10. Torres B, Aguilar F, Franco E, Sanchez E, Sanchez-Roman J, Jimenez Alonso J, et al. Association of the CT60 marker of the CTLA4 gene with systemic lupus erythematosus. Arthritis Rheum. 2004 Jul;50:2211-5.
11. Yanagawa T, Taniyama M, Enomoto S, Gomi K, Maruyama H, Ban Y, et al. CTLA4 gene polymorphism confers susceptibility to Graves' disease in Japanese. Thyroid. 1997 Dec;7:843-6.
12. Allahabadia A, Heward JM, Nithiyananthan R, Gibson SM, Reuser TT, Dodson PM, et al. MHC class II region, CTLA4 gene, and ophthalmopathy in patients with Graves' disease. Lancet. 2001 Sep 22;358:984-5.
13. Kouki T, Gardine CA, Yanagawa T, Degroot LJ. Relation of three polymorphisms of the CTLA-4 gene in patients with Graves' disease. J Endocrinol Invest. 2002 Mar;25:208-13.
14. Furugaki K, Shirasawa S, Ishikawa N, Ito K, Kubota S, Kuma K, et al. Association of the T-cell regulatory gene CTLA4 with Graves' disease and autoimmune thyroid disease in the Japanese. J Hum Genet. 2004;49:166-8.
15. Ban Y, Concepcion ES, Villanueva R, Greenberg DA, Davies TF, Tomer Y. Analysis of immune regulatory genes in familial and sporadic Graves' disease. J Clin Endocrinol Metab. 2004 Sep;89:4562-8.
16. Ban Y, Tozaki T, Taniyama M, Tomita M. Association of a CTLA-4 3' untranslated region (CT60) single nucleotide polymorphism with autoimmune thyroid disease in the Japanese population. Autoimmunity. 2005 Mar;38:151-3.
17. Weng YC, Wu MJ, Lin WS. CT60 single nucleotide polymorphism of the CTLA-4 gene is associated with susceptibility to Graves' disease in the Taiwanese population. Ann Clin Lab Sci. 2005 Summer;35:259-64.
18. Petrone A, Giorgi G, Galgani A, Alemanno I, Corsello SM, Signore A, et al. CT60 single nucleotide polymorphisms of the cytotoxic T-lymphocyte-associated antigen-4 gene region is associated with Graves' disease in an Italian population. Thyroid. 2005 Mar;15:232-8.
19. Han SZ, Zhang SH, Li R, Zhang WY, Li Y. The common -318C/T polymorphism in the promoter region of CTLA4 gene is associated with reduced risk of ophthalmopathy in Chinese Graves' patients. Int J Immunogenet. 2006 Aug;33:281-7.
20. Chen PL, Fann CS, Chang CC, Wu IL, Chiu WY, Lin CY, et al. Family-based association study of cytotoxic T-lymphocyte antigen-4 with susceptibility to Graves' disease in Han population of Taiwan. Genes Immun. 2008 Mar;9:87-92.
21. Tsai ST, Huang CY, Lo FS, Chang YT, Tanizawa T, Chen CK, et al. Association of CT60 polymorphism of the CTLA4 gene with Graves' disease in Taiwanese children. J Pediatr Endocrinol Metab. 2008 Jul;21:665-72.

22. Takahashi M, Kimura A. HLA and CTLA4 polymorphisms may confer a synergistic risk in the susceptibility to Graves' disease. J Hum Genet. 2010 May;55:323-6.
23. Zhao SX, Pan CM, Cao HM, Han B, Shi JY, Liang J, et al. Association of the CTLA4 gene with Graves' disease in the Chinese Han population. PLoS One. 2010;5:e9821.
24. Chistiakov DA, Turakulov RI. CTLA-4 and its role in autoimmune thyroid disease. J Mol Endocrinol. 2003 Aug;31:21-36.
25. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003 May 29;423:506-11.
26. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982 Nov;25:1271-7.
27. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988 Feb 11;16:1215.
28. Heward J, Gordon C, Allahabadia A, Barnett AH, Franklyn JA, Gough SC. The A-G polymorphism in exon 1 of the CTLA-4 gene is not associated with systemic lupus erythematosus. Ann Rheum Dis. 1999 Mar;58:193-5.
29. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007 Sep;81:559-75.
30. Dupont WD, Plummer WD, Jr. Power and sample size calculations. A review and computer program. Control Clin Trials. 1990 Apr;11:116-28.
31. Matsushita M, Tsuchiya N, Shiota M, Komata T, Matsuta K, Zama K, et al. Lack of a strong association of CTLA-4 exon 1 polymorphism with the susceptibility to rheumatoid arthritis and systemic lupus erythematosus in Japanese: an association study using a novel variation screening method. Tissue Antigens. 1999 Dec;54:578-84.
32. Liu MF, Wang CR, Lin LC, Wu CR. CTLA-4 gene polymorphism in promoter and exon-1 regions in Chinese patients with systemic lupus erythematosus. Lupus. 2001;10:647-9.
33. Lee YH, Kim YR, Ji JD, Sohn J, Song GG. Polymorphisms of the CTLA-4 exon 1 and promoter gene in systemic lupus erythematosus. Lupus. 2001;10:601-5.
34. Cho HJ, Chung JH, Kim IS, Kim HJ, Cho SH, Ki CS, et al. Lack of a genetic association between the CTLA-4 gene and Graves' disease in Koreans. Thyroid. 2006 Mar;16:237-41.
35. Kouki T, Sawai Y, Gardine CA, Fisfalen ME, Alegre ML, DeGroot LJ. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. J Immunol. 2000 Dec 1;165:6606-11.
36. Ban Y, Davies TF, Greenberg DA, Kissin A, Marder B, Murphy B, et al. Analysis of the CTLA-4, CD28, and inducible costimulator (ICOS) genes in autoimmune thyroid disease. Genes Immun. 2003 Dec;4:586-93.
37. Maurer M, Loserth S, Kolb-Maurer A, Ponath A, Wiese S, Kruse N, et al. A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (CTLA4) gene (exon 1 +49) alters T-cell activation. Immunogenetics. 2002 Apr;54:1-8.
38. Ulker M, Yazisiz V, Sallakci N, Avci AB, Sanlioglu S, Yegin O, et al. CTLA-4 gene polymorphism of exon 1(+49 A/G) in Turkish systemic lupus erythematosus patients. Int J Immunogenet. 2009 Aug;36:245-50.
39. Oaks MK, Hallett KM, Penwell RT, Stauber EC, Warren SJ, Tector AJ. A native soluble form of CTLA-4. Cell Immunol. 2000 May 1;201:144-53.
40. Kavvoura FK, Akamizu T, Awata T, Ban Y, Chistiakov DA, Frydecka I, et al. Cytotoxic T-lymphocyte associated antigen 4 gene polymorphisms and autoimmune thyroid disease: a meta-analysis. J Clin Endocrinol Metab. 2007 Aug;92:3162-70.