行政院國家科學委員會專題研究計畫 期中進度報告

葛瑞夫茲氏病遺傳研究-連鎖及關聯研究(2/3)
期中進度報告(完整版)

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行政院國家科學委員會補助專題研究計畫 期中進度報告

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中文摘要

葛瑞夫茲氏病是常見的自體免疫疾病，發病原因一般認為是多重因素，也與遺傳有關。我們過去的連鎖研究涵蓋 122 個葛瑞夫茲氏病家族，共 536 個人。所有家族皆有兩位以上的兄弟姊妹罹患此病，若有可能，父母之檢體也涵蓋在內。若父母之一或兩者之血液檢體不能取得，則要包含一位未罹病之兄弟姊妹之檢體。共計有 270 對罹患葛瑞夫茲氏病之兄弟姊妹(affected sib-pairs)可以做無母數連鎖分析(non-parametric linkage study)。我們從白血球萃取 DNA，並做 short tandem repeat polymorphism(STRP)標記之基因定型。我們選擇涵蓋 HLA 區 13.7 cM 之 8 個 STRP，平均每個標記的密度為 1.9 cM。多點無母數連鎖分析顯示與 HLA 區有相關，尖峰在標記 UniSTS:239159(LOD score 3.46, P = .00003, NPL Z score 4.1, P = .00002) (Clin Endocrinol 2007; 66:646-651)。在 CTLA4 方面，我們的連鎖研究涵蓋 151 個葛瑞夫茲氏病家族，包括 374 個病人和 347 個沒病的家人。在 CTLA4 基因定型 4 個 single nucleotide polymorphisms (SNP)和一個 STRP。我們新發現葛瑞夫茲氏病與 5’上游區的 SNP CTLA4_-1722_T/C (rs733618)相關(P = 0.0096)。我們也重現 SNP，CTLA4_+49_G/A (rs231775) ，與葛瑞夫茲氏病相關(P = 0.0219) 。一個共通的 haplotype，由 CTLA4_-1722_T/C 和 CTLA4_(AT)n (STRP 標記:UniSTS:48500) 組合而成，顯示保護的效果 (P = 0.0004)。我們在家族的連鎖研究，和白種人的結果，均顯示 CTLA4 與葛瑞夫茲氏病相關，是跨種族的(Gene and Immunity, 2008; 9:87-92).。在這次以族群為基礎的研究，我們已完成 1026 位檢體收集。其中有 1024 位病人確診為葛瑞夫茲氏病。包括男性 176 位，女性 848 位 (男比女 = 1:4.8) 。平均年齡為 40.9 ± 12.8 歲 (範圍 9 – 81 歲)。發病平均年齡為 35.9 ± 12.6 歲 (範圍 6 – 81 歲)。甲狀腺腫程度為 2.1 ± 0.9 (範圍 0 – 4)。有 500 位病人(48.8%)有眼病變(Gr. 1:126, 12.3%; Gr 2:234, 22.9%; Gr. 3:140, 13.7%)。33 位病人(3.2%)有脛前粘液水腫。19 位病人(1.9%)有週期性癱瘓。13 位病人(1.3%)有重症肌無力。

7 位病人(0.7%) 有白斑。我們已用 Affymetrix 500K chip 在 246 位有眼病變之女性葛瑞夫茲氏病病人做基因定型，並和 224 位女性對照組及 468 位男性及女性對照組做 genomewide association study 。結果顯示 HLA 區之 SNPs 有明顯之統計意義(-Log10(p)=5.841) 。我們進一步在 721 位家族檢體及 500 位族群病人做 HLA 基因定型，等取得中研院 500 位對照組做 HLA 基因定型後統計分析，希望能找到與葛瑞夫茲氏病相關之基因。
關鍵詞：葛瑞夫茲氏病，連鎖研究，關聯研究，基因

英文摘要

Graves' disease (GD) is an autoimmune disorder. The etiology of GD is accepted to be multifactorial with genetic effect. Our linkage study in the past, included 536 individuals in 122 multiplex families. All the families contained at least two affected siblings. Parents were also enrolled whenever possible; if samples from one or both parents were unavailable, at least one additional unaffected sibling was included. These participants could be analyzed as 270 affected sib-pairs (ASPs) in non-parametric linkage study. Genomic DNA was extracted from peripheral leukocytes. Genotyping of short tandem repeat polymorphism (STRP) markers was performed. Eight STRPs in a 13.7 cM region covering the HLA were chosen, resulting in a 1.9-cM (average) marker density. Multi-point non-parametric linkage analysis yielded evidence of significant linkage to the HLA region, which peaked around the marker UniSTS:239159 (LOD score 3.46, P = .00003; NPL Z score 4.1, P = .00002) (Clin Endocrinol 2007; 66:646-651). For CTLA4 study, we enrolled 374 affected individuals and 347 unaffected family members in 151 GD pedigrees. Four single nucleotide polymorphisms (SNP) and a short tandem repeat polymorphism (STRP) at CTLA4 were genotyped. Association of GD with a novel risk SNP at the 5' upstream region, CTLA4_-1722_T/C (rs733618), was demonstrated (P = 0.0096). We also replicated the association signal of a coding SNP, CTLA4_+49_G/A (rs231775, P = 0.0219). A common haplotype composed of CTLA4_-1722_T/C and CTLA4_(AT)n (a STRP marker:UniSTS:48500) showed protective effect (P = 0.0004). Our results of family-based association study, taken together with those from the Caucasian population, provide evidence that CTLA4 confers susceptibility to GD across different ethnic backgrounds (Gene and Immunity, 2008; 9:87-92). In the present population-based association study, we have completed the collection of

blood of 1,026 individuals. There are 1024 persons confirmed to be GD, which include 176 men, and 848 women (men to women =1:4.8). The mean age is 40.9 $\pm$ 12.8 years (range 9 – 81 years). The mean age of onset of hyperthyroidism is 35.9 $\pm$ 12.6 years (range 6 – 81 years). The degree of goiter is 2.1 $\pm$ 0.9 (range 0 – 4). There are 500 patients (48.8%) with ophthalmopathy (Gr. 1: 126, 12.3%; Gr 2: 234, 22.9%; Gr. 3: 140, 13.7%), 33 patients (3.2%) with pretibial myxedema, 19 patients (1.9%) with periodic paralysis, 13 patients (1.3%) with myasthenia gravis, 7 patients (0.7%) with vitiligo. Genotyping with Affymetrix 500K chip was performed in 246 female patients of Graves' disease with ophthalmopathy. We compared with two kinds of control samples **:**Control I, females only, N = 224**;** Control II, both males and females, N = 468. The genomewide association study showed the SNPs at HLA region were highly significant (-Log10(p)=5.841). The HLA genotype data of all the individuals (721 familial samples and 500 population-based samples) are completed, but 500 control samples are waiting from Academia Sinica. We will try to find the susceptibility genes at the HLA region.

Key words: Graves' disease, Linkage study, Association study, Gene

Linkage of Graves' disease to the human leucocyte antigen region in the Chinese-Han population in Taiwan (Clin Endocrinol 2007; 66:646-651)

INTRODUCTION

Graves' disease (GD [MIM 275000], http://www.ncbi.nlm.nih.gov/Omim/) is a common autoimmune disorder characterized by hyperthyroidism, diffuse goiter, thyroid-specific auto-antibodies, with or without ophthalmopathy and dermopathy.[1] Its prevalence is estimated to be around 1.0 to 1.6% in the general population.[2] The etiology of GD is generally accepted to be multifactorial[1,3] with strong evidence of a genetic effect, including family clustering,[4] an increased sibling risk (λs) of approximately 8 to 15,[4,5] and a higher concordance rate in monozygotic as compared to dizygotic twins (0.35 vs. 0.03).6 Data from 8,966 Danish twin pairs have suggested that 79% of the predisposition to GD is attributed to genetic factors.[6] The lack of a clear inheritance pattern implies that multiple genes are involved in the pathogenesis of GD.[7] Previous linkage analysis and association study results have implicated many genomic regions including the HLA region, the cytotoxic T-lymphoctye-associated 4 (CTLA4) gene, and the protein tyrosine phosphatase-22, that might harbor susceptibility genes for GD.[8-10]

The HLA region on chromosome 6p21 contains many important immune response genes. A number of population-based genetic association studies supported the association between the HLA region and GD.[11-18] Nevertheless the associated genes/alleles have not been consistent across multiple populations and negative studies were also published.[8, 9, 19] On the other hand, family-based studies have been more controversial. Only one linkage analysis in Caucasians demonstrated a nominal linkage with an NPL score = 1.95.[20] Yet other linkage studies in families from the US, Tunisia, Japan and China have not shown linkage to the HLA region.[21-26] The apparent discrepancy between association studies and linkage analysis, and the discrepancy between different populations, make the HLA region still an intriguing candidate for additional testing.[8] However, it should also be noted that differences between HLA allele and haplotype frequencies have been observed in different ethnic backgrounds.[27-30] It is therefore not uncommon that the disease-associated HLA haplotypes are not the same across populations.[31] Linkage analysis using family samples have suggested more than 20 different loci that might harbor susceptibility genes of GD and/or autoimmune thyroid disease (AITD).[8, 9] On the basis of these reports and their biological relevance, we also investigated four other candidate regions in our first attempt for linkage analysis: the *CTLA4* region on chromosome 2q33;[20] the cytokine gene cluster region on 5q31;[24, 25] the *pendrin* region on 7q22;32

and the *GD-1* and the thyroid stimulating hormone receptor regions on 14q31 in addition to the HLA region.[25] Here we report
significant linkage of GD to the HLA region on chromosome 6p21 with a non-parametric LOD score of 3.46 and a NPL Z score of 4.1, but not to the other 6 candidate regions.

MATERIALS AND METHODS

Subjects

Pedigrees were ascertained through a GD proband attending the outpatient clinic of National Taiwan University Hospital or affiliated clinics, Far Eastern Polyclinic. All the individuals enrolled in this study were interviewed and assessed by board-certified endocrinologists. The diagnosis of GD was made based on thepresence of biochemical hyperthyroidism together with either the presence of thyroid eye disease or a diffuse goiter and a significant titer of
auto-antibodies (including anti-microsomal, anti-thyroglobulin or anti-TSH receptor antibody) as previously reported.[19] To enrich phenotypic homogeneity, pedigrees containing any member with
possible Hashimoto's thyroiditis (HT [MIM603372]), either according to medical records of HT or self-stated history of symptoms or signs of hypothyroidism without previous thyroidectomy or radioactive iodine treatment, were not included in this study. Ethnic background was recorded according to the information from these individuals. Only subjects whose four grandparents were of Chinese Han origin were included, whereas those with ancestors of possible Taiwanese aboriginal (of Pacific-Polynesian extraction) or other minority Chinese ethnicity were not. This project was approved by the Institutional Review Board of National Taiwan University Hospital. Written informed consent was obtained from each individual.

This study included a total of 536 individuals in 122 multiplex families. All the families contained at least two affected siblings. Parents were also enrolled whenever possible; if samples from one or both parents were unavailable, at least one additional unaffected sibling was included. Among the pedigrees, 73 had two, 32 had three, 9 had four, 3 had five, 4 had six and 1 had eight affected individuals in one family. The pedigrees included a total of 321 affected patients, including 254 females (79.1%) and 67 males (20.9%). Of the 215 unaffected individuals, 113 were females and 102 males. These participants could be analyzed as 270 affected sib-pairs (ASPs) in
non-parametric linkage study.

Short tandem repeat polymorphism (STRP) markers selection and genotyping

Genomic DNA was extracted from peripheral leukocytes using the PureGene kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's

protocol. Fluorescence-labeled primers were purchased from Applied Biosystems (Foster City, CA, USA). Genotyping of STRP markers was performed on an ABI PRISM 3100 Genetic Analyzer, with allele calling done by Genotyper Software v 3.7 (Applied Biosystems). Each genotype was independently reviewed by two members. Mendelian inconsistency was checked with PedCheck (version 1.1).[33] Those genotypes with initial Mendelian inconsistency were rechecked, were corrected ifobvious mistake was identified, or set as missing. All the genotypes reported here were compatible with Mendelian inheritance. The overall genotype call rate was 97.5%.

Eight STRPs (D6S1660- D6S1691- D6S276- D6S273- UniSTS:239159-D6S1568- D6S291- D6S1610) in a 13.7 cM region covering the HLA were chosen, resulting in a 1.9-cM (average) marker density. The genetic positions of the markers were determined using the Marshfield (Center for Medical Genetics) genetic maps (http://research.marshfieldclinic.org/genetics/), and the order was verified with the physical map of National Center for Biotechnology Information (NCBI) build 35 (http://www.ncbi.nlm.nih.gov/). One marker (UniSTS:239159) was chosen from UniSTS database in NCBI build 35 without the information in the Marshfield geneticmaps. Its genetic position was approximated based on the physical distances between
flanking markers.

The other 26 markers distributed in the other four candidate regions were as followings: five markers (D2S118- D2S2387- UniSTS:48500- D2S155- D2S2242) in a 15.1-cM region on 2q33, seven markers (D5S2017- D5S436- D5S2090- D5S434-D5S2014- D5S410- D5S422) in an 18.9-cM region on 5q31, five markers (D7S2446-D7S501- D7S496- D7S2459- D7S486) in a 10.1-cM region on 7q22, and nine markers (D14S276- D14S274- D14S63- D14S258- D14S74- D14S1044- D14S280-D14S1054- D14S65) in a 61.1-cM region on 14q31. The necessary sequence information for primer design was based on the database in NCBI. The information of primers is available upon request.

Statistical analysis

Non-parametric linkage analyses were performed to locate the position of the susceptibility genes for GD. The allele frequency of markers was estimated on the basis of founders' genotypes. MERLIN 1.0.1. program was used to carry out two-point and multi-point nonparametric linkage analyses.[34] The non-parametric linkage (NPL) Z score and non-parametric LOD score under an exponential model were calculated.[35, 36] The information content of the genotypes was estimated with use of entropy information.[35] The Sall scoring function was used to capture the information of the allele sharing between all affected individuals in a pedigree.[37] The

1-LOD support interval was based on our multi-point non-parametric LOD scores.

RESULTS

Our familial collection comprised 270 ASPs (all possible pairs) for the Sall scoring function of non-parametric linkage analysis. For the five promising candidate regions, the maximal multi-point NPL Z scores calculated with MERLIN 1.0.1. program were as followings: 0.97 on chromosome 2q33, 0.82 on chromosome 5q31, 4.1 on chromosome 6p21, -0.89 on chromosome 7q22, and -0.34 on chromosome14q31 (Table 1).

Non-parametric analysis demonstrated linkage of GD to the HLA region onchromosome 6p21. Two-point analysis with MERLIN showed the highest non-parametric LOD score of 2.56 (P = 0.0003) at D6S1568 (Table 2). Multi-point non-parametric LOD score peaked around marker UniSTS:239159, but not exactly at the location of the marker (Figure 1), with the highest score of 3.46 (P =0.00003). The score specifically for this marker is 3.44 (Table 2)**.** Multi-point NPL Z score correlated very well with the LOD score (Table 2), peaking at the same UniSTS:239159 marker (maximal NPL Z = 4.1, P = 0.00002). Our 1-LOD support interval was a ~4.5 cM region (44.6 cM – 49.1 cM, sex-average distance) on theMarshfield genetic map (Figure 1). This 1-LOD support interval corresponds to a ~8.9 Mb region (26.9 Mb – 35.8 Mb) on the NCBI build 35 physical map, which contains the whole ~4.0 Mb HLA region (Figure 1).

DISCUSSION

Our results strongly support linkage of GD to the HLA region on chromosome 6p21. Among previous five studies of familial linkage analysis, the only positive signal at the HLA region was reported from the United Kingdom, with a nominal level of significance with an NPL score =1.95.[20] The other linkage studies, using pedigrees from the United States, United Kingdom, Japan, China, and Tunisia did not detect significant effect at the HLA region.[21-26] However, the results from various population-based association studies also offer support for the roles of HLA as a genetic contributor to GD.[7-9]

In this study, we attempted to decrease genetic heterogeneity, one major problem in genetic studies.[38, 39] We enrolled only pedigrees with Chinese-Han ethnic background. We also try to reduce the genetic heterogeneity by excluding families with a history of hypothyroidism or HT, although it could not be possibly complete. There are both advantages and disadvantages of our
decision to focus on GD. This strategy obviously would negatively influence our sample size, and thus decreased our power to detect susceptibility genes with effect

on both GD and HT. However, although GD and HT may share some common pathophysiological pathways, these two diseases have substantial differences in terms of clinical manifestations, laboratory abnormality and histological findings.[40] Previous reports have suggested that there might be different sets of susceptibility genes for GD, HT and AITD.[21, 22] In a recent large-scale genome-wide linkage screen with 1,119 AITD relative-pairs, none of the linkages obtained from GD or autoimmune hypothyroidism (AIH) was the same.[26] In addition, the diagnostic definition of HT is more controversial,[8] and the etiology of HT may be even more heterogeneous than GD.[21] In fact, the clinical course is variable in HT, and thyroid function could be normal or abnormal (overt hypothyroidism, subclinical hypothyroidism, and hyperthyroidism).[41] The measurement of anti-thyroglobulin and thyroid peroxidase antibodies can not provide any help in distinguishing various types of AITD.[42]

Our 1-LOD support interval was a ~4.5 cM region (44.6 cM–49.1 cM, sex-average distance) on the Marshfield genetic map, which corresponds to an ~8.9 Mb region (26.9 Mb – 35.8 Mb) on the NCBI build 35 physical map. This interval contains the whole ~4 Mb HLA region.[43] Class II loci, especially the HLA DRB1*03 and the DRB1*03-DQB1*02-DQA1*0501 haplotype, have repeatedly demonstrated to associate with GD in Caucasian populations.[8, 19, 44] However, in studies of populations of Chinese, Thai, Japanese and Korean ancestry, the DRB1*03 and its haplotype did not show association with GD; instead, there were reports for association with class I and other class II loci.[8, 14, 18, 44, 45] One plausible explanation is that different associated haplotypes reported in different populations actually carry some common polymorphisms driving the same autoimmune response. Due to the extended haplotype spectrum of the HLA genes, it has been extremely difficult to identify the primary etiological variants or to "split" the effect of individual loci from the effect of the whole haplotype.[19, 44] Comparison between association studies from populations with different composition of HLA haplotypes may provide very useful clues. Our result provides strong evidence for the involvement of the HLA region in GD in the Chinese-Han population in Taiwan, which advocates for the importance of a larger association study to help clarify the susceptibility HLA loci and/or alleles. Besides, it is still possible that some genes, other than the classical HLA loci, account for our linkage and the previous association signals. The recent linkage-disequilibrium map at the HLA region will facilitate the interpretation of future association studies.[27, 43] In addition, Simmonds et al. recently using various statistical techniques were able to dissect the effect of individual loci from the effects of neighboring variants, all associated with GD because of strong linkage-disequilibrium in this region.[19] All these recent progresses offer the hope for

eventual identification of the primary etiological genetic variants for GD in the future. We also tested the linkage of GD to **four other** candidate regions, including the *CTLA4* region on chromosome 2q33,[20] the cytokine gene cluster region on 5q31,[24, 25] the *pendrin* region on 7q22,[32] and the *GD-1* and the *TSHR* regions on 14q31.[25] The average marker density at different regions varied from 1 marker per 2.5 cM to 1 marker per 7.6 cM. None of the NPL Z scores reached suggestive level of significance, although we did see some positive signal at 2q33 (maximal NPL Z score = 0.97) and 5q31 (maximal NPL Z score = 0.82). Our results can not exclude any of the four regions, as the lack of signals could
be due to power limitation because of the underlying gene effects and our modest sample size and marker density. Linkage analysis has had great success in genetic mapping of **monogenic** diseases. Unfortunately, the power for detecting linkage signal of loci with moderate or small effect, the most possible situation in complex traits, is limited.46-48 Risch's calculation demonstrates that it takes more than 3000 affected sib-pairs to have sufficient power of detecting linkage signal of a susceptibility locus with genotype risk ratio of 2, while less than 1000 cases and controls are needed to find association using case control studies.[46] Therefore, further studies are necessary for these regions.

In conclusion, our work provides strong support of linkage of GD to the HLA region. Taken together with the previous linkage report in Caucasians and other association studies, our results suggest that the HLA region harbors one or more susceptibility genes for GD. Further dissection of this region in our population may provide insight into the immunogentics of GD pathogenesis, therefore is warranted.

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Table 1 Maximal Multi-Point NPL Z Scores at the Five Candidate Chromosome Regions

REGION	MARKER[a]	LOCATION[b] (cM)	Maximal NPL Z[c]	*P*=
2q33	D2S2319	210.43	0.97	0.2
5q31	D5S422	164.19	0.82	0.2
6p21	UniSTS:239159[d]	46.34	4.10	0.00002
7q22	D7S2446	113.92	-0.89	0.8
14q31	D14S274	63.25	-0.34	0.6

[a] The marker with the highest NPL Z score within the respective region.

[b] Genetic map locations were determined using sex-average distance on the Marshfield genetic map.

[c] Non-parametric Linkage (NPL) Z score calculated with the MERLIN 1.0.1. program.

[d] UniSTS:239159 is not included in the Marshfield genetic map. Its genetic map location was approximated based on physical distances between flanking markers.

Table 2 The Results of Non-Parametric Linkage Analysis at the HLA Region on 6p21

MARKER	LOCATION		TWO-POINT ANALYSIS			MULTI-POINT ANALYSIS				
	Genetic Map[a] (cM)	Physical Map[b] (Mb)	LOD[c]	*P*	Info. Content	LOD[c]	*P*	NPL Z[d]	*P*	Info. Content
D6S1660	40.14	23.4	0.04	0.3	0.51	1.45	0.005	2.44	0.007	0.86
D6S1691	42.27	24.0	1.36	0.006	0.76	1.69	0.003	2.80	0.003	0.94
D6S276	44.41	24.3	0.35	0.1	0.60	2.32	0.0005	3.31	0.0005	0.95
D6S273	44.96	31.8	0.48	0.07	0.61	2.64	0.0002	3.53	0.0002	0.95
UniSTS:239159[e]	46.34	33.3	1.39	0.006	0.72	**3.44**	**0.00003**	**4.10**	**0.00002**	**0.96**
D6S1568	47.71	34.1	**2.56**	**0.0003**	**0.76**	3.32	0.00005	3.87	0.00005	0.95
D6S291	49.50	36.3	0.94	0.02	0.63	1.30	0.007	2.44	0.007	0.92
D6S1610	53.81	39.4	0.82	0.03	0.67	1.37	0.006	2.41	0.008	0.85

[a] Genetic map locations were determined using sex-average distance on the Marshfield genetic map.
[b] Physical map locations were determined using NCBI human genome map build 35.
[c] Non-parametric LOD score based on the exponential model calculated with the MERLIN 1.0.1. program.
[d] NPL Z score calculated with the MERLIN 1.0.1. program.
[e] The UniSTS:239159 marker is not included in the Marshfield genetic map. Its genetic map location was approximated based on physical distances between flanking markers.

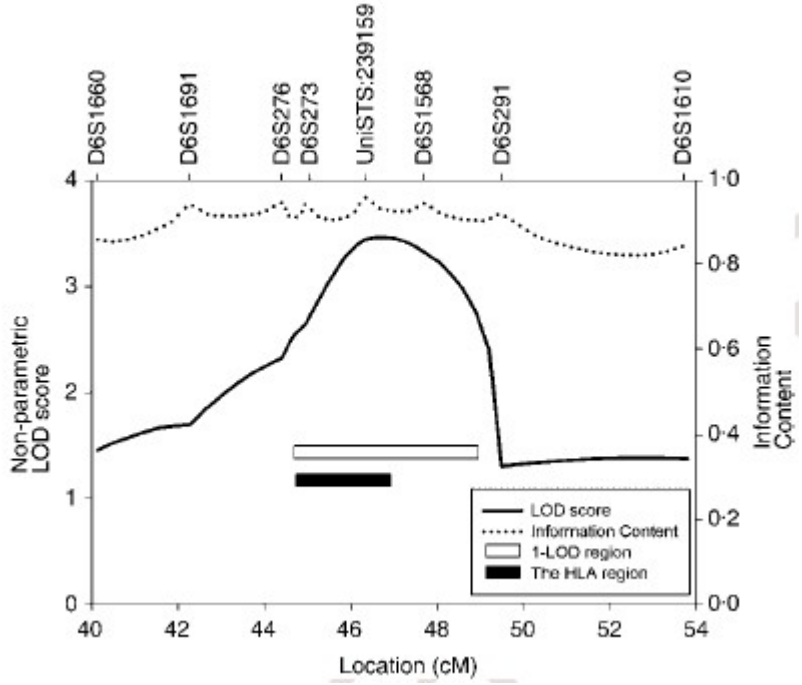

Figure 1 Multi-point non-parametric LOD scores on 6p21-p22 from linkage analysis of 270
ASPs in 122 GD families. The scores were calculated with MERLIN v 1.0.1. Multi-point
non-parametric LOD scores (solid line), information content (dotted line), the 1-LOD region
(empty bar) and the HLA region (filled bar) are plotted. The X-axis values are distances from
the p-telomere, in Kosambi cM.

Family-based association study of cytotoxic T-lymphocyte antigen-4 with susceptibility to Graves' disease in the Chinese-Han population in Taiwan (Gene and Immunity, 2008; 9:87-92)

Introduction

Graves' disease (GD) is a common organ-specific autoimmune disorder manifested with hyperthyroidism, diffuse goiter, thyroid-specific antibodies, with/without ophthalmopathy and/or dermopathy (1). The prevalence of GD in the general population was estimated to be around 1.0 to 1.6% (2-4). The cause of GD is multifactorial, with considerable genetic influence (1, 5). The evidence of the genetic contribution to its pathogenesis came from the observation of family clustering (6), an increased risk ratio between 8 and 15 of patients' female siblings (6, 7), and a higher concordance rate in monozygotic twins (0.35) than in dizygotic twins (0.03) (8). A statistic model based on the data from 8,966 pairs of Danish twins demonstrated that nearly 80% of the predisposition to GD could be attributed to genetic factors (8). However, being short of a common Mendelian inheritance pattern suggests a polygenic nature in its pathogenesis (9, 10).

Cytotoxic T lymphocyte antigen 4 (*CTLA4*) is an immunoregulatory molecule expressed on the surface of T lymphocytes and serves as a key negative regulator (11). *CTLA4* gene located on chromosome 2q33 is among the most plausible candidate genes for GD (10, 12, 13). Many population-based association studies in different ethnic backgrounds have shown positive results (10), although negative results have also been reported (14, 15). The most frequently reported association signals are CTLA4_+49_G/A (rs231775, at exon 1), CTLA4_-319_C/T (rs5742909, at the promoter region) and CTLA4_(AT)n (UniSTS:48500, a STRP marker at the 3' untranslated region of *CTLA4*) (10, 12, 13). Ueda *et al.* reported a comprehensive SNP fine mapping and proposed that the SNPs at the 3' uncoding region, such as CTLA4_CT60_G/A (rs3087243, at the 3' downstream region), are the primary disease determinants (16). Nevertheless, more studies are probably needed to reveal the genuine causative genetic variants.

In contrast to the population-based studies, there have been few family-based genetic studies providing evidence of the involvement of *CTLA4*. Up to now, however, only three family-based studies in the United Kingdom observed linkage or association signal of GD at the *CTLA4* gene (16-18). Population-based genetic association studies in outbred populations may be prone to false positives because of population stratification (19, 20), which can be avoided by family-based design (21). In this study we enrolled 721 individuals in 151 pedigrees with one or more affected persons in the Chinese-Han population in Taiwan. Family-based association study

was performed with 5 markers previously reported to be associated with GD or other autoimmune diseases. We found that a novel risk SNP of GD at the 5' upstream region of *CTLA4* (rs733618, named as CTLA4_-1722_T/C in this report) and haplotypes containing this SNP are significantly associated with GD.

Results

Genotypes of four SNPs (CTLA4_-1722_T/C, CTLA4_-1147_C/T, CTLA4_+49_G/A and CTLA4_CT60_G/A) and a dichotomized STRP marker (CTLA4_(AT)n_O/S) were analyzed with TRANSMIT v2.54 (22) for family-based association study. The basic information of these markers and allele frequency in our samples were summarized in Table 1. The allele frequencies of all these SNP markers were compatible with Hardy-Weinberg equilibrium. The relative positions of these markers at *CTLA4* gene were shown in Figure 1. For single marker analysis, our strongest association signal was observed at a 5' upstream SNP, CTLA4_-1722_T/C, with its minor allele (C allele) over-transmitted to affected individuals (χ^2 = 6.714, df = 1, nominal P = 0.0096, Table 1). Our study is the first one to demonstrate association of CTLA4_-1722_T/C with GD. We also replicated the association signal at another SNP, CTLA4_+49_G/A (χ^2 = 5.252, df = 1, nominal P = 0.0219, G allele over-transmitted).

Linkage disequilibrium (LD) between these 5 markers was analyzed with Haploview 3.32 (23). We applied the "solid spine" option to define LD blocks. All the five markers were shown to be grouped into a single LD block (Figure 1), which is compatible with previous reports (16, 24). We applied TRANSMIT to perform family-based association analysis using haplotypes from all the possible 2-marker combinations. The result is presented in Table 2. Eight of the ten combinations had at least one haplotype with a nominal P value less than 0.05. The most significant cluster was related to CTLA4_-1722_T/C; all of its four 2-marker combinations had at least one haplotype with nominal P value less than 0.05, and three of its four 2-marker combinations had one haplotype with nominal P value less than 0.01. Our strongest 2-marker association signal was from a common haplotype (34.5% of the population) composed of the CTLA4_-1722_T allele and the CTLA4_(AT)n_O allele (Chi square = 12.576, df = 1, P = 0.0004). This haplotype was under-transmitted to the affected individuals, which indicated a protective role.

Discussion

We report an association of *CTLA4* with GD in a family-based study in the Chinese-Han population in Taiwan. Previously, the only three family-based researches on the association between *CTLA4* and GD were all performed in

Caucasians (16-18). Family-based study design has the advantage of overcoming possible spurious positives caused by population stratification (19-21). Our result provides robust evidence of the association between *CTLA4* and GD in the Chinese-Han population.

Our strongest association signals were observed at a novel risk SNP, CTLA4_-1722_T/C, and haplotypes containing it. This SNP was previously reported to be associated with systemic lupus erythematosus (SLE) in two contradictory studies; the T allele was reported to increase risk in Koreans (25) while the C allele was reported in a Spanish study (26). In a recent association study of celiac disease in the Irish population (27), CTLA4_-1722_T/C itself did not reach statistically significant association but some haplotypes containing the CTLA4_-1722_T allele were shown to be protective. In our study, the T allele of CTLA4_-1722_T/C SNP and haplotypes containing it were under-transmitted to the affected individuals. We conclude that the T allele of CTLA4_-1722_T/C SNP, or other genetic variant(s) in close linkage disequilibrium with it, has protective effect for GD in the Chinese-Han population.

Our most significant result was found in a 2-marker combination containing CTLA4_-1722_T/C and CTLA4_(AT)n markers (P = 0.0004). CTLA4_(AT)n has been shown associated with GD in a couple of studies (12, 28, 29), with the "allele 106" of Yanagawa *et al.* study (28) as the risk allele. The length of the dinucleotide expansion was shown to lead to T cell hyper-reactivity in myasthenia gravis (30). Ueda *et al.* (16) dichotomized this STRP marker for single-locus and two-locus association study in their comprehensive SNP fine mapping. In our current report, we dichotomized this STRP marker into "allele S" (standing for the "Specific" allele which is the same as the "allele 106" of Yanagawa et al. study (28)) and "allele O" (standing for the "Other" alleles). The direction of allele effect was found to be consistent between previous reports ("allele 106" as the risk allele) and our study ("allele O" to be in the protective haplotype).

We also notice that the CTLA4_(AT)n marker is not in strong LD with other markers, judging from the measurement of D' (Figure 1a) and R^2 (Figure 1b). One explanation for the highly significant association signal from the two-marker combination is that a single causal genetic variant was not genotyped in our study and its information could be captured by the haplotypes defined by these two markers. However, another possible explanation is that there are two (or even more) different causal variants at the *CTLA4* gene, and CTLA4_-1722_T/C and CTLA4_(AT)n represented independent signals. Ueda *et al.*, with the statistic model attempting to detect a single causal variant at *CTLA4*, proposed that one SNP at the 3' uncoding region is the primary disease determinant (16). However, their data

turned out unable to exclude the possibility of multiple causative variant(s). We suggest further exploration of the two-causal-variants hypothesis with more samples and markers in the future.

In addition to being compatible with the two-causal-variants hypothesis, our results further raise an intriguing possibility that those two causative variants have some kind of interaction. The P value from the single marker analysis of our association study for CTLA4_(AT)n was 0.22, which did not provide evidence of significant effect. However, when the combination of CTLA4_-1147_T and CTLA4_(AT)n_O alleles were tested, the P value (0.0004) was 24-fold smaller than that of CTLA4_-1147_C/T SNP (P = 0.0096) alone. The observation is remarkably reminiscent of the Ueda *et al.* study, in which this STRP marker itself showed only very modest single locus association but was one of the few markers that could further increase the association signal under their best model containing the CTLA4_CT60_G/A SNP. We propose that there are two causative variants working together to exert maximal influence on the gene. Replication from other independent samples and evidence from functional studies are necessary to prove this hypothesis.

Our study should be considered as a hypothesis-testing approach because all of the 5 markers have previously been reported to be associated with GD or other autoimmune diseases. This kind of approach affects our analysis in two major ways. First, this set of markers are not tagging markers, which means that further exhaustive haplotype analyses (3-marker, 4-marker and 5-marker haplotype analysis) may not give us interpretable results. The current single-marker and two-marker analyses can be viewed as tests for individual effect and joint (or interaction) effect of these candidate markers. Secondly, it will be very difficult to adapt an appropriate method to correct for multiple testing. Having been previously reported of association, these markers actually have a higher *a priori* to be the true signal; being in linkage disequilibrium with each other and inside the same LD block (Figure 1), the penalty for multiple testing should be reduced (31). Therefore in this manuscript we only report the nominal P values and leave the issue of correction for multiple testing untouched. Nonetheless, it is obvious that our best association signals remain significant even under the most stringent Bonferroni correction.

By comparing our genotypes of the unrelated founders and those in public database, we noticed allele frequency difference across populations of a couple of SNPs at the *CTLA4* gene region. For example, the frequency of the CTLA4_CT60_A allele in our samples was 20.9%, which is very close to that of the Chinese Han in Beijing (CHB) samples of the HapMap project (21.1%) (32), but very different from that of the Utah residents with ancestry from northern and western Europe (CEU) samples of the HapMap project (45.8%). Similarly, the allele

frequency of CTLA4_-1722_C allele in our samples was 42.6%, which is comparable with the CHB samples (33.3%) but differs significantly from the CEU samples (5.8%). Figure 2 demonstrates the allele frequency of CTLA4_-1722_T/C, CTLA4_+49_G/A and CTLA4_CT60_G/A. It intrigues us to find such big allele frequency difference in some specific SNPs which have been repeatedly tested for association with GD and/or other autoimmune diseases. It is hard to say if this kind of difference indicates any biological meaning. However, the allele frequency difference of these three SNPs between Caucasian samples and our Chinese Han samples was very significant. It might partially explain why the association study of *CTLA4* gave inconsistent results. We suggest that any attempt of applying the risk (or protective) alleles/haplotypes information at *CTLA4* across populations should be done with great caution.

With the same collection of familial samples, we did not detect evidence of linkage at the *CTLA4* region (33), which could be explained by the effect size of *CTLA4* and by the sample size of our collection. However, we were able to demonstrate significant linkage (NPL score = 4.1, P = 0.00002) at the human leukocyte antigen (HLA) region (33). With an intention to reduce heterogeneity of our samples, we purposely enrolled individuals with pure Chinese-Han ethnic background, and collected GD-only pedigrees by excluding families with a history of hypothyroidism or Hashimoto's thyroiditis. With this strategy of sample collection, we successfully detected linkage signal of HLA and association signal of *CTLA4*. It is likely that this collection may contribute our genetic study at other regions in the future.

In conclusion, our family-based association study demonstrates that the *CTLA4* gene is associated with GD in the Chinese-Han population in Taiwan. We identified a novel risk SNP in the 5' flanking region (CTLA4_-1722_T/C, P = 0.0096). A two-marker combination composed of the CTLA4_-1722_T allele and the CTLA4_(AT)n_O allele showed protective effect (P = 0.0004). Our results of family-based association study, taken together with those from the Caucasian population, provide robust evidence that *CTLA4* confers susceptibility to GD across different ethnic backgrounds.

Subjects and Methods

Clinical assessment and family ascertainment:

Pedigrees were ascertained through a GD proband as previously described (33). Briefly, the diagnosis of GD was made based on the manifestations of biochemical hyperthyroidism with either ophthalmopathy or a diffuse goiter and a significant titer of auto-antibodies, including anti-microsomal, anti-thyroglobulin or anti-TSH

receptor antibody as in previous reports (33, 34). To increase phenotypic homogeneity, pedigrees containing any member with possible Hashimoto's thyroiditis (HT [MIM603372], based either on medical records of HT or on self-stated history of symptoms/signs of hypothyroidism without previous treatment of thyroidectomy or radioactive iodine) were excluded. To reduce heterogeneous ethnic background, only subjects with four grandparents of Chinese-Han origin were included. Those patients with ancestors of possible Taiwanese aboriginal (of Pacific-Polynesian origin) or other minority Chinese ethnicity were not enrolled. This project was approved by the Ethic Committee of Human Research of National Taiwan University Hospital. Written informed consent was obtained from each individual.

This report included 721 individuals in 151 pedigrees. Among the pedigrees, 6 (4.0 %) had one affected individual, 100 (66.3 %) had two, 28 (18.5 %) had three, 8 (5.3 %) had four, 7 (4.6 %) had five, 1 (0.7 %) had seven and 1 (0.7 %) had 10 affected individuals in one family. There were 374 affected patients, including 300 females (80.2 %) and 74 males (19.8 %). Of the 347 unaffected family members, 156 were females and 191 males.

Genotyping:

Genomic DNA was extracted from peripheral leukocytes using the PureGene kit following the protocol from the manufacturer (Gentra Systems, Inc., Minneapolis, MN, USA). Four SNPs at the *CTLA4* gene region were genotyped, including CTLA4_-1722_T/C (rs733618), CTLA4_-1147_C/T (rs16840252), CTLA4_+49_G/A (rs231775) and CTLA4_CT60_G/A (rs3087243)(Table 1). Genotyping was performed using LightCycler (Roche Diagnostics, Basel, Switzerland) or a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-based method with MassARRAY (Sequenorm, Inc., San Diego, CA, USA) at the genotyping core facility at the Academia Sinica in Taipei. The sequence information for primer and probe design was based on the public genome database (http://www.ncbi.nlm.nih.gov). To genotype the STRP marker CTLA4_(AT)n, fluorescence-labeled primers were purchased from Applied Biosystems. PCR was performed in a 10-μl reaction volume containing 25 ng of genomic DNA and 1 unit of AccuPrime Taq polymerase (Invitrogen Corp., Carlsbad, CA, USA) according to the standard protocol. After PCR, 3 μl of the product was mixed with 0.5 μl of internal size standard and 10 μl of deionized formamide, denatured, and separated using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Allele calling was performed by using Genotyper Software v 3.7 (Applied Biosystems). Each genotype was independently reviewed by two members of the research team. The sequences of primers and probes are available upon request.

Statistical analyses:

Mendelian inheritance consistency of all markers in individual pedigree was checked with PedCheck version 1.1 (35) to confirm the family structure and genotyping correctness. Those genotypes with initial Mendelian inconsistency were rechecked, and corrected if obvious mistake was identified, or set as missing. All the pedigrees included in this report are compatible with Mendelian inheritance. For all the 4 SNPs, Hardy-Weinberg equilibrium (HWE) was also checked using the genotype frequency of unrelated founders. None of the SNPs reported in this study was against HWE.

We applied TRANSMIT v2.54 software (22) to conduct family-based association study. TRANSMIT adapts a score test based on a partial score function that omits the terms most influenced by hidden population stratification. In a region without evidence of linkage, TRANSMIT can handle uncertainty for multilocus haplotypes, allow parental genotypes to be unknown and deal with more than one affected offspring per family. Therefore it is appropriate for our study. Haploview 3.32 (23) was used to analyze linkage disequilibrium (LD) between markers. The "solid spine" option was applied to define LD blocks.

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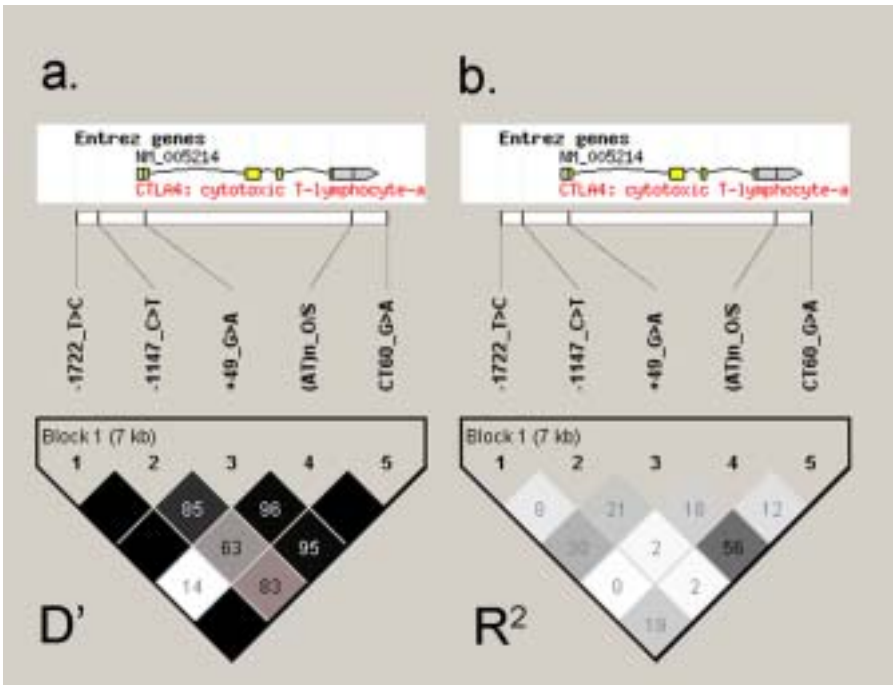
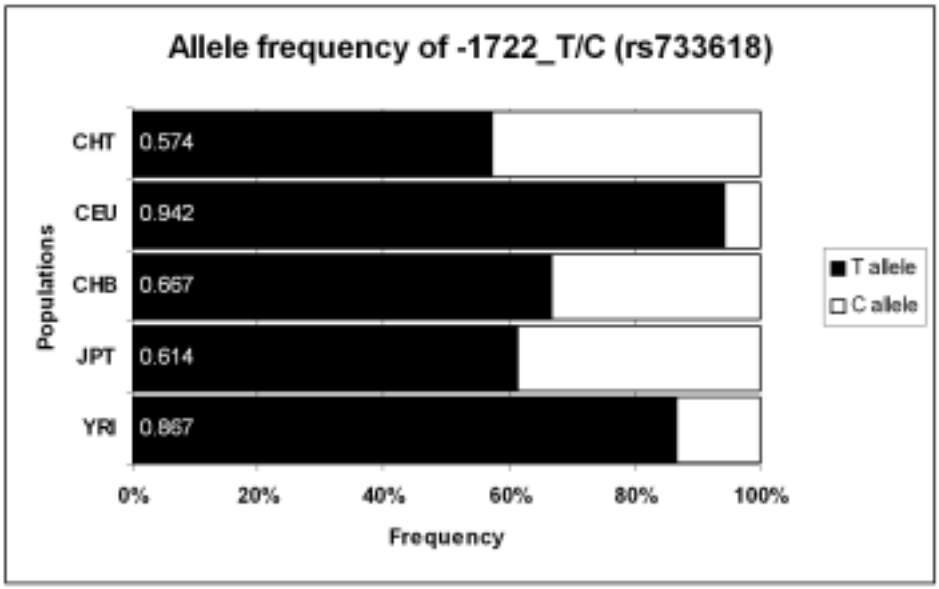
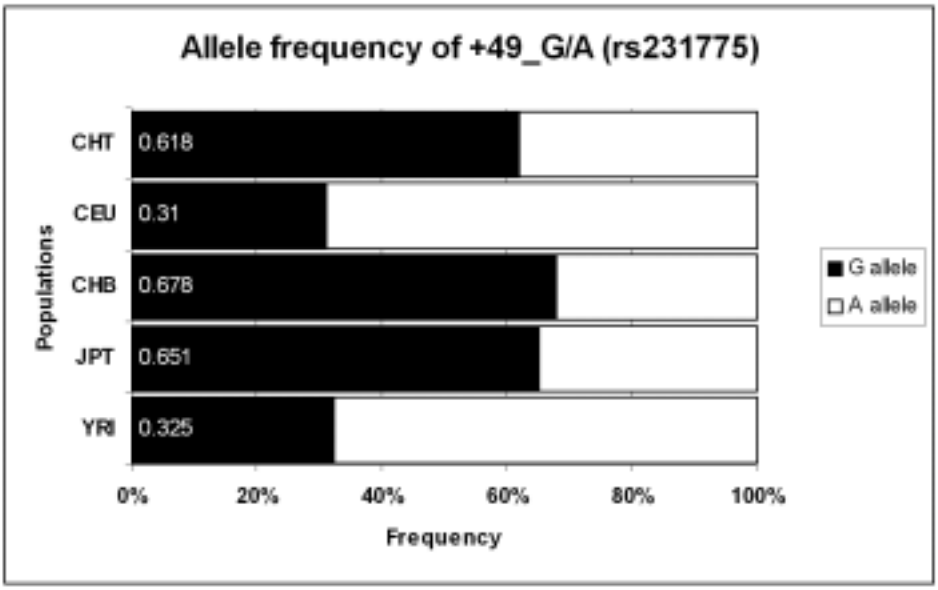
Figure legend:

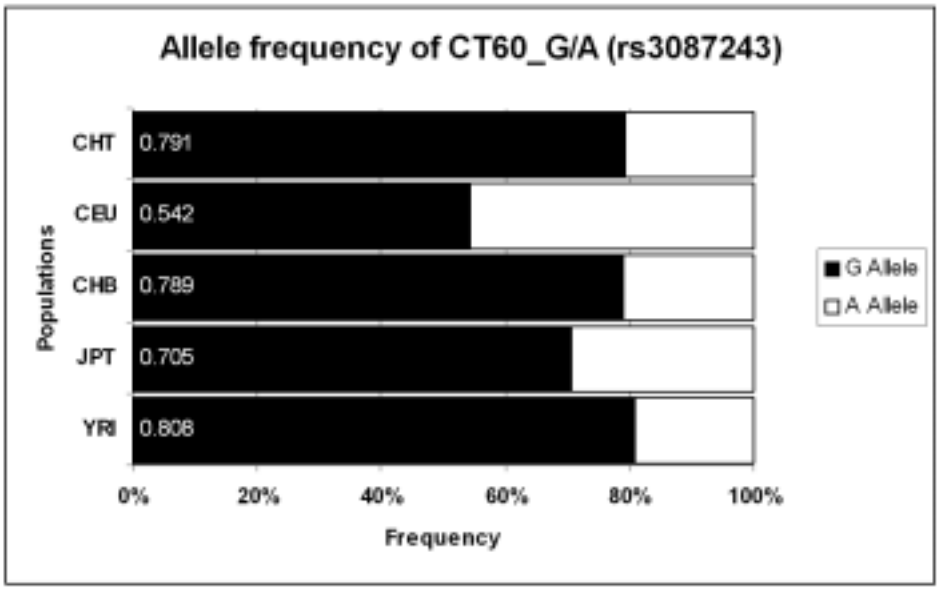
Figure 1. Pairwise LD pattern of markers at *CTLA4* measured by (a) D' and (b) R^2. These five markers were grouped as a single block spanning 7 kb, using the "solid spine" block definition of Haploview (23)v3.32. The location of each tested marker relative to *CTLA4* is indicated on top. The number in each diamond indicates the magnitude of LD in percent between respective pairs of markers. For example, the pairwise LD for -1722_T/C and (AT)n_O/S is 0.14 in D' and 0 in R^2. Diamonds without values are the ones with D' equal to 100%.

Figure 2. Allele frequency of three SNPs in different populations. Abbreviation of the populations: **CHT**: Chinese Han in Taiwan (our current collection); **CEU**: CEPH (Utah residents with ancestry from northern and western Europe); **CHB**: Chinese Han in Beijing; **JPT**: Japanese in Tokyo; and **YRI**: Yoruba in Ibadan, Nigeria. The allele frequency for CHT came from our study using the genotypes of unrelated founders. All the other allele frequency was from the HapMap project (32), except for the allele frequency of +49_G/A of CEU which was from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html) because of the lack of information in HapMap.




Allele frequency of -1722_T/C (rs733618)
CHT
CEU
CHB
JPT
YRI
0.574
0.942
0.667
0.614
0.867
Populations
0%
20%
40%
60%
80%
100%
Frequency
T allele
C allele


Allele frequency of +49_G/A (rs231775)
CHT
CEU
CHB
JPT
YRI
0.618
0.31
0.678
0.651
0.325
Populations
0%
20%
40%
60%
80%
100%
Frequency
G allele
A allele


Allele frequency of CT60_G/A (rs3087243)
CHT
CEU
CHB
JPT
YRI
0.791
0.542
0.789
0.705
0.808
Populations
0%
20%
40%
60%
80%
100%
Frequency
G Allele
A Allele

Tables:

Table 1. Information and 1-marker association test of the 4 SNPs and 1 STRP marker

Marker Name	Name, NCBI Build 36.1	Frequency of Minor Allele	Chi square, (df 1) TRANSMIT[b]	*P* value, TRANSMIT[b]
CTLA4_-1722_T/C	rs733618	C allele, 0.427	+ 6.71[c]	**0.0096**[d]
CTLA4_-1147_C/T	rs16840252	T allele, 0.111	+ 3.08	0.079
CTLA4_+49_G/A	rs231775	A allele, 0.288	- 5.25	**0.0219**
CTLA4_(AT)n_O/S[a]	UniSTS:48500	S allele, 0.358	+1.50	0.22
CTLA4_CT60_G/A	rs3087243	A allele, 0.199	- 2.45	0.11

[a]CTLA4_(AT)n_O/S is a dinucleotide (AT) STRP marker. "S" allele stands for the "Specific" allele which is the same as the "allele 106" of Yanagawa et al. study (28), and "O" allele stands for the collection of all the "Other" alleles.

[b]TRANSMIT v. 2.54 (22), with the option –ro (to use the robust estimate of the variance of the score vector).

[c]The sign in this column is the sign of Chi square statistics of the minor allele. The plus sign means over-transmission of the minor allele, and the minus sign under-transmission.

[d]Nominal P value less than 0.05 is marked as bold letters

Table 2. 2-marker association test: all possible combinations

Marker name	Haplotype	Freq.	Chi-quare (df 1), TRANSMIT	P value, TRANSMIT
-1722_T/C, -1147_C/T	T:C	0.465	-2.23[a]	0.1350
	C:C	0.422	+7.34	**0.0067**
	T:T	0.111	-4.36	**0.0369**
	C:T	0.002	+1.70	0.1919
-1722_T/C, +49_G/A	C:G	0.429	+5.38	**0.0204**
	T:A	0.291	-7.27	**0.0070**
	T:G	0.280	+0.04	0.8328
-1722_T/C, (AT)n_O/S	T:O	0.345	-12.58	**0.0004**
	C:O	0.302	+3.76	0.0524
	T:S	0.229	+0.75	0.3867
	C:S	0.124	+2.10	0.1474
-1722_T/C, CT60_G/A	C:G	0.426	+5.86	**0.0155**
	T:G	0.375	-0.79	0.3755
	T:A	0.199	-3.63	0.0568
-1147_C/T, +49_G/A	C:G	0.699	+7.70	**0.0055**
	C:A	0.188	-4.56	**0.0327**
	T:A	0.104	-2.38	0.1227
	T:G	0.010	-0.06	0.8078
-1147_C/T, (AT)n_O/S	C:O	0.541	-0.19	0.6661
	C:S	0.345	+2.40	0.1215
	T:O	0.103	-2.02	0.1556
	T:S	0.011	-1.88	0.1698
-1147_C/T, CT60_G/A	C:G	0.692	+6.26	**0.0123**
	C:A	0.196	-2.13	0.1448
	T:G	0.110	-3.16	0.0755
	T:A	0.002	-1.33	0.2486
+49_G/A, (AT)n_O/S	G:O	0.369	+0.33	0.5669
	G:S	0.348	+2.69	0.1010
	A:O	0.284	-5.32	**0.0211**
	A:S	0.007	-1.17	0.2800
+49_G/A, CT60_G/A	G:G	0.702	+3.92	**0.0477**
	A:A	0.193	-2.49	0.1149
	A:G	0.100	-0.94	0.3317
	G:A	0.005	-0.02	0.8781
(AT)n_O/S, CT60_G/A	O:G	0.445	+0.07	0.7899
	S:G	0.355	+1.20	0.2737
	O:A	0.198	-3.75	0.0528
	S:A	0.002	+1.92	0.1654

[a]The sign in this column is the sign of Chi square statistics of this haplotype. The plus sign means over-transmission of this haplotype, and the minus sign under-transmission.

Genowide Association Study

In the present population-based association study, we have completed the collection of blood of 1,026 individuals. There are 1024 persons confirmed to be GD, which include 176 men, and 848 women (men to women =1:4.8). The mean age is 40.9 $\pm$ 12.8 years (range 9 – 81 years). The mean age of onset of hyperthyroidism is 35.9 $\pm$ 12.6 years (range 6 – 81 years). The degree of goiter is 2.1 $\pm$ 0.9 (range 0 – 4). There are 500 patients (48.8%) with ophthalmopathy (Gr. 1: 126, 12.3%; Gr 2: 234, 22.9%; Gr. 3: 140, 13.7%), 33 patients (3.2%) with pretibial myxedema, 19 patients (1.9%) with periodic paralysis, 13 patients (1.3%) with myasthenia gravis, 7 patients (0.7%) with vitiligo. Genotyping with Affymetrix 500K chip was performed in 246 female patients of Graves' disease with ophthalmopathy. We compared with two kinds of control samples **:**Control I, females only, N = 224**;** Control II, both males and females, N = 468. The genomewide association study showed the SNPs at HLA region were highly significant (-Log10(p)=5.841). The HLA genotype data of all the individuals (721 familial samples and 500 population-based samples) are completed, but 500 control samples are waiting from Academia Sinica. We will try to find the susceptibility genes at the HLA region.

Affymetrix GeneChip® Mapping 500K Array

There are 489,922 SNPs on 22 chromosomes

~ 500,000 SNPs in the 3*109 bp human genome.

"On average" 6 kb per SNP, (the SNPs are NOT evenly distributed.)

Exclusion Criteria

1. non-polymorphism in both of case & control
2. HWE test (Hardy-Weinberg equilibrium test, p-value < 5*10-7) in both of case & control
3. MAF(minor allele frequency) < 5% in control
4. CallRate < 95% in combined case and control samples to make sure the quality of genotypes is good

About 317,000 SNPs were used for further analyses

SAS/Genetics was applied for single-point association test

Results

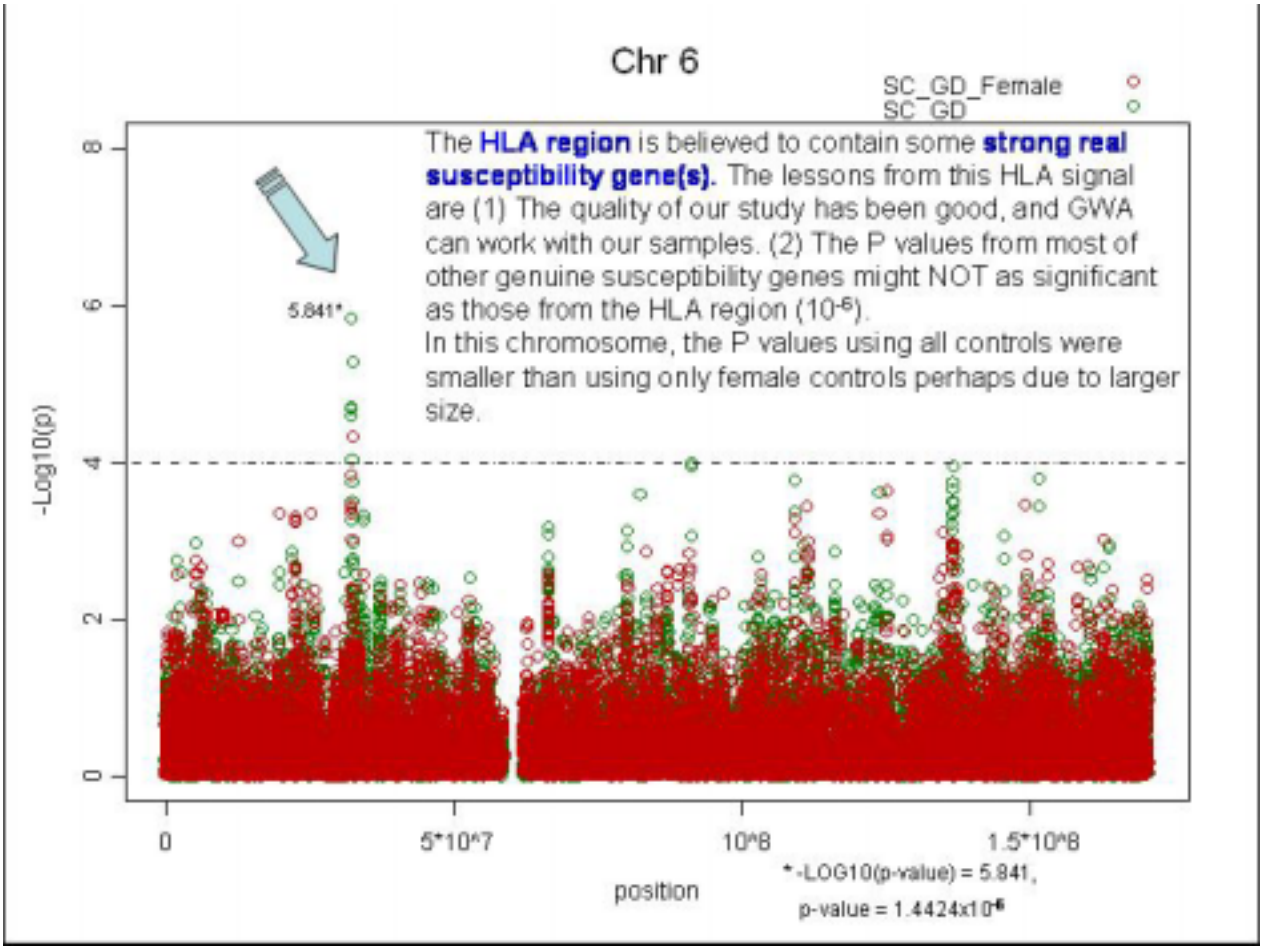
In chromosome 6, the **HLA region** is believed to contain some **strong real susceptibility gene(s).** The lessons from this HLA signal in our study are (1) The quality of our study has been good, and GWA can work with our samples. (2) The P values from most of other genuine susceptibility genes might NOT as significant as those from the HLA region ~ 10^{-6}. . In this chromosome, the P values using all controls were smaller than using only female controls perhaps due to larger size.

There are **5 SNPs in a ~ 120 kb** region in chromosome 1, with the best P value ~ 10^{-5}. The P values using all controls are smaller than those using female-only controls.

All of the 5 SNPs have good genotyping quality.

In chromosome 5, the results are more significant when using female-only controls (than all controls). GD, like several other autoimmune diseases, is predominantly found in females. **It is possible that some risk genes might be gender-specific.**

In chromosome 12, the region contains **two genes**; the function of these genes is poorly understood at this moment. Interestingly, one gene is highly expressed in B lymphocytes and T lymphocytes, and the other in B lymphocyte, monocyte and dendritic cells.



Association of HLA-A, HLA-B, HLA-C and HLA-DP, DQ, DR, to GD in the Chinese-Han population

Aim, Methods

Find the susceptibility alleles/loci/haplotypes of classical HLA genes to GD

Compare the risk alleles/loci/haplotypes between Chinese-Han and Caucasians

In our genotyping results, there were 15 HLA-A alleles, 45 HLA-B alleles, 22 HLA-C alleles, 18 HLA-DP alleles, 16 HLA-DQ alleles and 30 HLA-DR alleles.

The HLA genotype data of all the individuals (721 familial samples and 500 population-based samples are completed, but 500 control samples are waiting from Academia Sinica.

- We adapted DNA based genotyping (NOT the old serology-based method).

- We can achieve 4-digit of resolution. (For example HLA-DRB1*0301).
- We basically use the sequence-specific oligo (SSO) probe hybridization methods, either LABType SSO system of One Lambda, or Reli SSO method of DYNAL. These two companies/platforms are among the most reliable ones in this field. Sequence-based genotype confirmation was performed if necessary.

We hope we can find the susceptibility genes at the HLA region.