行政院國家科學委員會專題研究計畫　成果報告

葛瑞夫茲氏病基因研究-以家族為基礎之關聯研究（3/3）

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摘要

葛瑞夫茲氏病是常見的自體免疫性甲狀腺疾病，其原因通常被認為是多重的，與遺傳有密切的關係，人類白血球抗原（HLA）一度被報告與高加索人有關，但從沒有在其他人種被報告過。

我們在漢人測試 HLA 區及 4 個候選區基因是否與葛瑞夫茲氏病有關。

這是一個以家族為主的無母數聯鎖分析，使用了 8 個 short tandem repeat polymorphism（STRP）標記，涵蓋了染色體 6p21 之 HLA 區，及其他 4 個候選區之 26 個 STRPS。

本研究共有 127 個家族，604 個人，其中包括 357 位病人（288 位女性，69 位男性），和他們的 247 位沒有罹病的親戚（122 位女性，125 位男性），可以分析的罹病的兄弟姊妹配對為 199 對。

多點無母數聯鎖（NPL）分數分析顯示在 HLA 區有顯著的聯鎖，尖峰在 UniSTS：239159（NPL 分數 3.98，P = 0.00004），其 1-LOD support 間隔涵蓋約 4Mb HLA 區。

我們的結果是第一個能重覆葛瑞夫茲氏病與 HLA 區有關的研究，與高加索人的研究結果合併看來，在許多人種裏面，HLA 區帶有葛瑞夫茲氏病之易感基因。

關鍵詞：葛瑞夫茲氏病，聯鎖分析，人類白血球抗原（HLA）

Abstract

Context: Graves' disease (GD) is a common autoimmune thyroid disorder inherited as a complex trait with strong genetic predisposition. The human leukocyte antigen (HLA) region was once reported to be linked to GD in Caucasians but has never been replicated despite multiple linkage studies in various ethnic backgrounds.

Objective: As a candidate region approach, we tested if the HLA region, and four other candidate regions, is linked to GD in the Chinese Han population.

Design: This is a family-based non-parametric linkage analysis, using 8 short tandem repeat polymorphism (STRP) markers covering the HLA region on chromosome 6p21, and other 26 STRPs in four other candidate regions.

Setting: A medical center and its affiliated outpatient clinics.

Patients or Other Participants: There were 604 individuals from 127 Chinese Han complex pedigrees in Taiwan, including 357 affected patients (288 females, 69 males) and their 247 unaffected relatives (122 females, 125 males), which could be analyzed as 199 affected sib-pairs.

Intervention(s): No.

Main Outcome Measure(s): Increased allele sharing among affected individuals in the same family.

Results: Multipoint non-parametric linkage (NPL) score analysis showed significant linkage to the HLA region, which peaked at the marker UniSTS:239159 (NPL score 3.98, $P = .00004$) with the 1-LOD support interval covering the ~4 Mb HLA region.

Conclusions: Our result is the first replication of linkage of GD to the HLA region. Taken together with the finding in the Caucasian population, it suggests that the HLA region harbors a susceptibility gene(s) for GD in multiple ethnic groups.

Key words: Graves' disease, linkage study, HLA

Introduction

Graves' disease (GD [MIM 275000]) is a common autoimmune disorder characterized by hyperthyroidism, diffuse goiter, thyroid-specific auto-antibodies, ophthalmopathy and/or dermopathy (1). Its prevalence is around 1.0 to 1.6% in the general population (2, 3). The etiology of GD is generally accepted to be multifactorial (1, 4) with strong evidence of genetic effect including family clustering (5), a λs of approximately 8 to 15 (5, 6), and a higher concordance rate in monozygotic as compared to dizygotic twins (0.35 vs. 0.03) (7). A statistic model based on the data from 8,966 Danish twin pairs suggested that 79% of the predisposition to GD is attributed to genetic factors (7). The lack of a clear inheritance pattern implies that multiple genes are involved in the pathogenesis of GD (8, 9). According to previous linkage analysis and association study result, many regions have been suggested to harbor susceptibility genes of GD (10, 11). However, only few regions, including the HLA region, the cytotoxic T-lymphoctye-associated 4 gene (*CTLA4* [MIM 123890]), and the protein tyrosine phosphatase-22 (*PTPN22*) have shown more consistent results (12-14).

The HLA region on chromosome 6p21 contains many important immune response genes. A number of population-based genetic association studies, especially in Caucasians, have supported the association between the HLA region and GD, although the associated genes/alleles have not been consistent across multiple populations (10, 15). On the other hand, family-based studies have provided much less support for the HLA region. Only one linkage

analysis in Caucasians (16) demonstrated a nominal linkage (NPL score = 1.95), and two family-based association studies (17, 18), also in Caucasians, reported significant association. Other linkage analysis studies in families from the US, Tunisia, Japan and China (19-25) have not shown significant linkage signal for GD to the HLA region. This 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 (11).

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) (10, 11). On the basis of these results and biological relevance, we also included four other candidate regions in our linkage analysis: the *CTLA4* region on chromosome 2q33 (16); the cytokine gene cluster region on 5q31 (24); the *pendrin* [MIM 605646] region on 7q22 (26); and the *GD-1* (24) and the thyroid stimulating hormone receptor (*TSHR* [MIM 603372]) regions on 14q31.

Subjects and Methods

Subjects

Pedigrees were ascertained through a GD proband attending the outpatient clinic of National Taiwan University Hospital or the affiliated clinics, Far Eastern Polyclinic. All the individuals enrolled in this study were interviewed and assessed by endocrinologists. The

diagnosis of GD was made if at least two of the following four criteria were met: (i) documented hyperthyroidism, (ii) diffuse goiter, (iii) ophthalmopathy and/or dermopathy, and (iv) TSH receptor-specific antibody (which is tested with TSH-binding inhibitory immunoglobulin assay). If an individual only had documented hyperthyroidism and diffuse goiter, a positive anti-thyroid antibody (such as anti-thyroglobulin antibody or anti-thyroid peroxidase antibody) was required to establish the diagnosis. Pedigrees containing any member with Hashimoto's thyroiditis (HT [MIM603372]), by the evidence of positive anti-thyroglobulin antibody and/or anti-thyroid peroxidase antibody, diffuse hypoechoic thyroid in the ultrasonography, lymphocytes and Hürthle cells in the fine-needle aspiration cytology, and no clear evidence of GD, were excluded from 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 possible Taiwanese aboriginals (of Pacific-Polynesian extraction) or other minority Chinese ethnic groups were not. This project was approved by the Institutional Review Board of National Taiwan University Hospital, and written informed consent was obtained from each individual.

This study included a total of 604 individuals in 127 multiplex families. All the families contained at least two affected siblings. Parents were also enrolled if possible; when samples from one or both parents were unavailable, at least one additional unaffected sibling was

included. Among the pedigrees, 69 had two, 34 had three, 14 had four, and 10 had at least five affected individuals in one family (table 1). There were 357 affected patients, including 288 females (80.7%) and 69 males (19.3%). In the 247 unaffected individuals, 122 were females and 125 males. These participants could be analyzed as 199 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 of the research team. The overall success rate of the genotyping was 97.5%. Mendelian inconsistency was checked with PedCheck version 1.1 (27). All the pedigrees included in this report were compatible with Medelian inheritance.

Eight STRPs (D6S1660- D6S1691- D6S276- D6S273- UniSTS:239159- D6S1568- D6S291- D6S1610) in a 13.7 cM region covering the HLA were chosen (table 2), 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, and the order was verified with the physical map of National Center for Biotechnology Information (NCBI) build 35. One (UniSTS:239159) of the eight markers was chosen from UniSTS database in NCBI build 35 without information in the Marshfield genetic maps; its genetic position was approximated based on physical distances between flanking markers. For the other four candidate regions, the distribution of these 26 markers was 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. Two-point analysis was conducted with GENEHUNTER version 2.1 (28). Multipoint non-parametric linkage (NPL) analyses were tested with two independent

methods. The first was GENEHUNTER version 2.1, in which we used the S_{all} statistic to capture the information about the allele sharing between all affected individuals in a pedigree. The second method was SIMWALK2 (29-31), which was developed using Markov chain Monte Carlo (MCMC) and simulated annealing algorithms.

Results

Non-parametric analysis with different methods consistently demonstrated linkage of GD to the HLA region on chromosome 6p21. Two-point analysis with GENEHUNTER showed the highest NPL score of 2.38 (P = .0078) at D6S1568 (table 2). While performing multipoint NPL analysis with GENEHUNTER program, linkage peaked at marker UniSTS:239159 (fig. 1) with a NPL score of 3.98 (P = .00004). Our 1-LOD support interval was a ~5.2 cM region (43.3 cM – 48.5 cM, sex-average distance) on the Marshfield genetic map (fig. 1), which can be converted to a ~11.6 Mb region (24.2 Mb – 35.8 Mb) on the NCBI build 35 physical map. Analysis using SIMWALK2 program also yielded similar result (table 2), with the highest –Log(P) value of 3.91 at the same marker (UniSTS:239159).

For the other four regions, the multipoint NPL scores calculated by GENEHUNTER program are summarized in table 3. The maximal NPL scores were 0.77 on chromosome 2q33, 1.18 on chromosome 5q31, -0.63 on chromosome 7q22, and -0.31 on chromosome 14q31.

Discussion

Our results strongly support linkage of GD to the HLA region on chromosome 6p21. Previously, the only linkage analysis with positive signal at the HLA region came from the United Kingdom, with a nominal level of significance (NPL score = 1.95) (16). The other linkage studies, using pedigrees from the United States (19, 24, 25), Japan (21), China (22, 32), and Tunisia (23), did not detect any significant effect. Even a recent genome-wide linkage screen with 1,119 AITD relative-pairs (302 GD relative-pairs) in the Caucasian population could not find linkage signal at the HLA region (20). Inadequate power due to the nature of linkage analysis could be one explanation for the discrepancy between association studies and linkage analysis, and for the inconsistent results in different populations (33). Our study included 604 individuals in 127 complex families (equivalent to 199 ASPs in the GENEHUNTER program), which, to our knowledge, is among the largest GD family collections for linkage analysis published to date.

In addition to collecting a large sample, we also attempted to decrease genetic heterogeneity, another major problem in genetic studies (34-36). We enrolled only pedigrees with Chinese Han ethnic background according to the family history stated by the participants themselves. We also took great care to define the disease phenotype as precisely as possible. All of the participants were interviewed by at least one of the six endocrinologists of the same medical center, and evaluated with the same diagnostic criteria. Although GD and

Hashimoto's thyroiditis (HT) may share some common pathophysiological pathways, these two diseases have substantial differences in terms of clinical manifestations, laboratory abnormality and histological findings. Previous reports (19, 21) also suggested that there might be different sets of susceptibility genes for GD, HT and autoimmune thyroid disease (AITD, containing both GD and HT). In a recent genome-wide linkage screen in 1,119 AITD relative-pairs, none of the linkages obtained from GD or HT was the same (20). In addition, the diagnostic definition of HT is more controversial (11), and the etiology of HT may be even more heterogeneous than GD (19). As an attempt to further decrease heterogeneity, we purposely concentrated our study on GD. Pedigrees with any member of possible HT were not included. However, we are aware that, despite all the effort to collect GD-only families, it might not be possible to exclude all the families with both GD and HT individuals.

Our 1-LOD support interval was a ~5.2 cM region (43.3 cM – 48.5 cM, sex-average distance) on the Marshfield genetic map, which corresponds to an ~11.6 Mb region (24.2 Mb – 35.8 Mb) on the NCBI build 35 physical map. This interval contains the whole ~4 Mb HLA region (37). Class II loci, especially the HLA DRB1*03 and the DRB1*03-DQB1*02-DQA1*0501 haplotype, have repeatedly demonstrated association with GD in Caucasian populations (11, 12, 15). However, in studies of populations of Chinese, 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 (11, 12).

Due to the extended haplotypes of the HLA genes, it has been extremely difficult to identify the primary etiological variants (15) or “split” the effect of individual loci from the effect of the whole haplotype (12) with samples just from a single ethnic background. Comparison between association studies from populations with different composition of HLA haplotypes may provide very useful clues. Since our result shows a strong linkage signal, a follow up family-based or population-based association study with a large sample size may help clarify the susceptibility HLA loci and/or alleles. Besides, it is still possible that some genes, other than the classical HLA loci, accounted for our linkage signal. The recent linkage-disequilibrium map at the HLA region (37) will facilitate a future association study.

We also tested linkage of GD to other four candidate regions, including the *CTLA4* region on chromosome 2q33, the cytokine gene cluster region on 5q31, the *pendrin* region on 7q22, and the *GD-1* and the *TSHR* regions on 14q31. 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 scores reached suggestive level of significance, although we did see some positive signal at 2q33 (maximal NPL = 0.77) and 5q31 (maximal NPL = 1.18).

In conclusion, our work is the first replication 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 across multiple ethnic groups.

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Web Resources:

Accession numbers and URLs for data presented herein are as follows:

NCBI Database, http://www.ncbi.nlm.nih.gov/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for GD, HT, *CTLA4*, *pendrin*, and *TSHR*)

Marshfield Clinic Research Foundation (Center for Medical Genetics), http://research.marshfieldclinic.org/genetics/

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Figure and Tables:

Table 1

Summary of Families

NO. OF AFFECTEDS	NO. OF FAMILY
2	69
3	34
4	14
5	7
6	1
9	1
10	1

Table 2

The Results of Linkage Analysis at the HLA Region on 6p21

MARKER	LOCATION		TWO-POINT ANALYSIS			MULTI-POINT ANALYSIS			
	Genetic Map[a] (cM)	Physical Map[b] (Mb)	GENEHUNTER			GENEHUNTER			SIMWALK
			NPL	Information Content	*P*	NPL	Information Content	*P*	-LOG (*P*)
D6S1660	40.14	23.4	0.24	0.51	.398	2.37	0.85	.008	2.10
D6S1691	42.27	24.0	1.63	0.79	.048	2.72	0.93	.003	3.04
D6S276	44.41	24.3	0.95	0.63	.162	3.22	0.93	.0006	3.72
D6S273	44.96	31.8	1.17	0.64	.115	3.43	0.93	.0003	3.83
UniSTS:239159[c]	46.34	33.3	1.60	0.73	.050	**3.98**	**0.95**	**.00004**	**3.91**
D6S1568	47.71	34.1	**2.38**	**0.76**	**.008**	3.76	0.94	.00009	3.08

D6S291	49.50	36.3	1.63	0.62	.048	2.37	0.90	.008	2.15
D6S1610	53,81	39.4	1.57	0.67	.054	2.34	0.83	.009	2.06

[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] 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.

Table 3

Maximal Multipoint NPL Scores (MLS) at All the Five Candidate Regions

REGION	MARKER[a]	LOCATION[b] (cM)	MLS[c]	*P*
2q33	UniSTS:48500[d]	199.35	0.77	.21
5q31	D5S422	164.19	1.18	.10
6p21	UniSTS:239159[d]	46.34	3.98	.00004
7q22	D7S2446	113.92	-0.63	.64
14q31	D14S274	63.25	-0.31	.52

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

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

[c] Multipoint NPL scores were calculated with GENEHUNTER version 2.1, using the S_{all} statistic.

[d] UniSTS:48500 and UniSTS:239159 are not included in the Marshfield genetic map. Their genetic map locations were approximated based on physical distances between flanking markers.

Figure 1

Multipoint NPL scores on 6p21-p22 from linkage analysis of 199 ASPs in 127 GD families. The scores were calculated with GENEHUNTER v 2.1. Multipoint NPL 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.