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

計畫類別：個別型計畫
計畫編號：NSC91-3112-B-002-003-
執行期間：91 年 05 月 01 日至 92 年 04 月 30 日
執行單位：國立臺灣大學醫學院內科

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報告類型：完整報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 7 月 2 日

附件一

中文摘要

葛瑞夫茲氏病是一種常見的具有器官專一性之自體免疫疾病。它屬於人類複雜性疾病，而好發於具有易感受性體質之個體。過去十年來有相當多的研究者投入心血，然而，究竟哪些基因是此疾病的易感受基因卻仍然眾說紛紜。CTLA4 是一個重要的免疫調節分子，而也一直被視為是葛瑞夫茲氏病易感受基因的可能標的，然而，到目前為止，所有在非高加索人種所做的家族性研究中，從來沒有找到 CTLA4 是本疾病基因的證據。為了釐清 CTLA4 在非高加索人種中是否具有遺傳學上的重要性，我們針對在台灣的漢民族做了葛瑞夫茲氏病易感受基因的家族研究。本研究共收集 154 個純粹的葛瑞夫茲氏病家族，內含 382 個罹病的個案以及 256 個未罹病之家族對照者，這是到目前為止本疾病全球最大的家族研究。我們以 CTLA4 為目標基因來做遺傳流行病學的檢驗，總共做了七個微衛星標幟（以 CTLA4 為中心，前後共 24cM 的距離）以及一個單一鹼基（位於第一個 exon）的基因定型。我們發現，無母數連鎖分析得到的最大多點性 NPL 值為 1.46（P = 0.06），位置就是在 CTLA4 上，而家族性相關研究也得到統計學上顯著的結果（P = 0.036）。我們的成果提供了很好的證據來支持 CTLA4 基因和葛瑞夫茲氏病在漢民族具有連鎖以及相關。再合併先前在高加索人種的研究結果，暗示了 CTLA4 和葛瑞夫茲氏病的關係可能橫跨各個種族。

關鍵詞: 葛瑞夫茲氏病，基因，CTLA4，家族研究，台灣漢民族

ABSTRACT

Graves' disease (GD), a common organ-specific autoimmune disorder with clinical importance, is a multifactorial disease and develops in genetically susceptible individuals. Despite much effort during the past decade, the susceptibility genes of GD are still uncertain. The cytotoxic T lymphocyte antigen-4 (CTLA4) is an important negative regulator of antigen-activated immune response, and is among the most possible susceptibility genes of GD. However, up to now, none of family-based studies in non-Caucasian populations show linkage or association between CTLA4 and GD. To clarify the role of CTLA4 to GD outside Caucasian population, we conducted this CTLA4 in Chinese-Han pedigrees in Taiwan. We enrolled 382 affected and 256 unaffected individuals in 154 pure GD families, which is the largest family dataset in the world. As a candidate gene approach, we typed 7 microsatellite markers spanning 24 cM around CTLA4 gene, and 1 SNP marker at exon 1. Non-parametric linkage analysis peaked at the CTLA4 3'UTR microsatellite with multipoint NPL score of 1.46 (P = 0.06). Family-based association test yielded a z statistic of 2.102 (P = 0.036). Our results provide evidence that CTLA4 is both linked to and associated with GD in Chinese-Han population in Taiwan. Combining the previous results from Caucasian population, CTLA4 may be a susceptibility gene of GD across different ethnic background.

Key words: Graves' disease, gene, CTLA4, family-based studies, Taiwanese

II

Introduction

Graves' disease (GD) is a common organ-specific autoimmune disorder characterized by hyperthyroidism, diffuse goiter, thyroid-specific antibodies, ophthalmopathy and/or dermopathy (1). Its prevalence is around 1.0-1.6% in the general population (2-4). The etiology of GD is generally accepted to be multifactorial (1, 5). There is strong evidence supporting the role of genetic effect, including family clustering (6), increased sibling risk ratio relative to the general population, ës, of about 8 to 15 (6, 7), and much higher concordance rate in monozygotic twins than in dizygotic twins (0.35 vs. 0.03) (8). 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 (8). The lack of a clear inheritance pattern suggests that multiple genes are involved in pathogenesis of GD (9, 10).

During the last decade, many investigators have focused on identifying the genetic contributors of GD (10). Population-based case-control studies ended up with inconsistent reports after testing various candidate genes, which included human leukocyte antigen (HLA) (11-14), TSH receptor (15, 16), T-cell receptor (17), cytotoxic T lymphocyte antigen-4 (CTLA4) (18-23), tumor necrosis factor (24, 25), and Vitamin D receptor (26), etc. Family-based linkage or association studies, even after three genome-wide scans of GD (27-29), were also frustrated by low reproducibility. Among the loci with positive results from family-based study (14, 27, 30-36), such as GD1, GD2, GD3, CTLA4, HLA, 18q21, Xp11, and 5q31, only CTLA4, HLA and GD2 could be replicated by another different research team .

The CTLA4 is an immunoregulatory molecule expressed on the surface of T lymphocytes, and serves as a key negative regulator for antigen-activated immune response (37). Its gene, located on chromosome 2q33, is among the most likely susceptibility genes of GD (10). Many population-based studies in different ethnic background showed association (18-21, 38-42), although some others reported the opposite (22, 23). Linkage or family-bsaed association study, on the contrary, provided much less support. Only two family-based studies in the UK (35, 36) observed linkage or association, while all the other researches in the US (27), Japan (28), China (29), and Tunisia (43) failed to detect the effect.

Here, from Chinese-Han population in Taiwan, we enrolled 638 individuals in 154 multiplex pedigrees, which is the largest family dataset of pure GD in the world. We tested CTLA4 as a candidate locus and performed non-parametric linkage analysis and family-based association study. Our result demonstrated that CTLA4 confers susceptibility to GD in Chinese-Han population in Taiwan.

Subjects and Methods

Clinical assessment and family ascertainment

All the individuals were interviewed and assessed by doctors specializing in endocrinology. The diagnosis of GD was made when at least two of the following four criteria were fulfilled:

documented hyperthyroidism, diffuse goiter, ophthalmopathy or dermopathy, and TSH receptor
antibody. After informed consent, blood samples were collected for DNA preparation and biochemical measurements, and questionnaires were obtained. This project was approved by the Institutional Review Board of National Taiwan University Hospital.

We focused on population of pure Chinese Han descent, and excluded pedigrees with aboriginal or foreign ancestry. We collected pure GD pedigrees, and excluded the whole families if any one of their individuals was affected with Hashimoto's thyroiditis. Pedigrees were ascertained through a GD proband attending the Outpatient Clinic of National Taiwan University Hospital or one of its cooperative clinic, Far Eastern Polyclinic.

Genotyping

Genomic DNA was extracted from 6 ml of blood with PureGene® kit (Gentra Systems Corp.) according to the standard protocol from the manufacturer.

For microsatellite genotyping, oligonucleotide primers for 7 microsatellite markers around CTLA4 were chosen from Applied Biosystems microsatellite panels (ABI PRISM® Linkage Mapping Set v2.5), or designed according to published sequences in the genome database (http://gdbwww.gdb.org/). Their names and relative location from centromeric side to telemeric side are: **D2S364** - 2.79cM - **D2S118** - 5.65cM - **D2S2387** - 2.99cM - **CTLA4** - 4.28cM - **D2S155** - 2.14cM - **D2S2242** - 5.37cM - **D2S2319**. Fluorescence-labeled primers were purchased from Applied Biosystems Corp.. PCR was performed in 10 ì l reaction volume containing 10 ng genomic DNA, 5 pmol of each primer, PCR buffer (50 mM KCL, 10 mM Tris-HCL with pH 8.3, 1.5 mM MgCl2, 200 ì M each of deoxy-NTPs), and 1 U of AccuPrime® Taq polymerase (Invitrogen Corp.). Reaction mixtures were heated at 95 C for 5 min, then cycled for 35 times with denaturation at 95 C for 30 sec, annealing at 55 C for 30 sec, and extension at 68 C for 30 sec, and followed by a final extension at 68 C for 10 min. After PCR, 3 ì l of the product was mixed with 0.5 ì l internal size standard and 10 ì l deionized formamide, denatured, and separated using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Corp.). Allele calling was performed using Genotyper® Software v 3.7. Each genotype was reviewed visually by two members

of the research team.

For SNP genotyping (CTLA4 49 A/G), we adapted the melting curve analysis system for mutation detection of LightCycler® (Roche Diagnostic Corp.).

Statistic analyses

For linkage analysis, we calculated non-parametric linkage (NPL) scores by using the multipoint algorithm in GENEHUNTER program (44) version 2.0. We used the ALL function, which examines all affected individuals simultaneously.

For association study, we used the family-based association test (FBAT) package (45). It provides a z statistic that tests the composite null hypothesis of no linkage or no disequilibrium. FBAT excel conventional transmission disequilibrium test (TDT) in being able to use data from all family members, not just case-parent trios.

Results

We enrolled 638 individuals in 154 pedigrees of Chinese Han population in Taiwan. Among the pedigrees, 6 (3.9 %) had one affected individual, 101 (65.6 %) had two, 30 (19.4 %) had three, 8 (5.2 %) had four, 7 (4.5 %) had five, 1 (0.7 %) have seven, and 1 (0.7 %) have ten affected individuals. In the total 638 individuals, there are 382 affected patients, including 307 female (80.1%) and 75 male (19.9%). In the 256 unaffected individuals, there are 125 female and 131 male.

Non-parametric linkage analysis was conducted with GENEHUNTER program. Multipoint NPL score peaked at CTLA4 marker (Fig. 1). The highest NPL score is 1.46 (P = 0.06) with borderline significance.

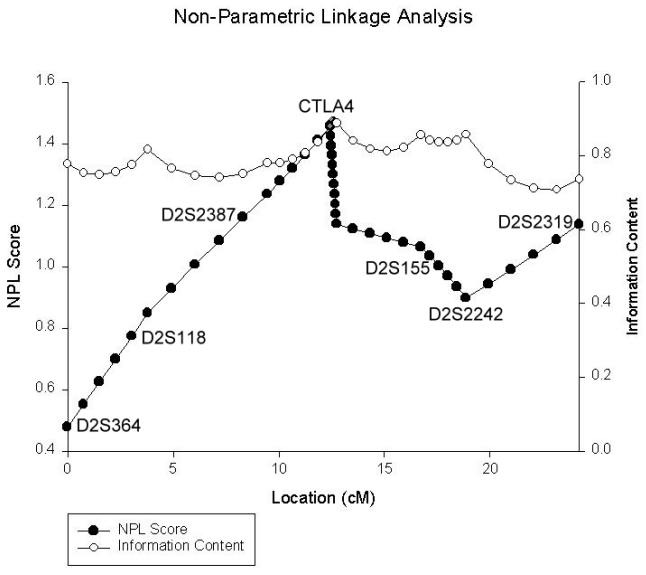

Fig. 1. Multipoint nonparametric linkage analysis of 7 markers around CTLA4. The NPL score (left Y-axis) is shown as solid circles against the marker map (X-axis). The information content (right Y-axis) is shown as empty circles. The maximum evidence for linkage, a NPL score of 1.46 (P = 0.06), occurs at CTLA4 marker.

Association study was performed with FBAT (family-based association test)

package. We applied candidate gene approach to test if the SNP at exon 1 (49 A/G) is associated with Graves' disease. The resultant z statistic is 2.102 (P = 0.036), which shows evidence for association between CTLA4 gene and Graves' disease.

Discussion

Based on the largest family dataset of GD in the world, our results provide evidence that CTLA4 confers susceptibility to GD. This is for the first time that this effect can be demonstrated in a family-based study in non-Caucasian population, which implies that CTLA4 is a susceptibility gene across different ethnic background. Before our project, only two family-based study in the UK (35, 36) showed linkage or association, while other researches based on pedigrees in the US (27), Japan (28), China (29), and Tunisia (43) failed to detect the effect. Family-based study is of paramount importance because it can eliminate false-positive results due to population stratification or other pitfalls stick to population-based approach (46-53). One reason for the irreproducibility is about the sample size (54), and the others may relate to strategy

about family ascertainment and study design (see next paragraphs). Our project has enrolled 638 individuals in 154 families, which is about 1.5 to 2-fold the sample size of other studies if only pure GD families were compared.

Our family dataset is also characterized by its homogeneity, pure GD pedigrees in Chinese-Han ethnic background. Genetic heterogeneity has long been regarded as a substantial obstacle in genetic study (50-53). One attempt to reduce heterogeneity is to draw samples from single ethnic group, which has been demonstrated useful in a meta-analysis study (55). Another reasonable attempt is to define the disease phenotype as precise as possible. Although GD and Hashimoto's thyroiditis (HT) might share some common pathophysiology pathways, these two diseases are substantially different in clinical manifestations, laboratory data and histological findings. Previous reports (27, 28) also suggested that there might be different sets of susceptibility genes for GD, HT and autoimmune thyroid disease (AITD, containing both GD and HT); and, HT might be even more heterogeneous than GD (27). We thus decided to focus on GD, and excluded all the pedigrees with aboriginal ancestry or containing HT patients. Our design further reduced the possible heterogeneity during ascertainment; all the individuals were assessed and recruited by one of four endocrinologists at our medical center.

Our design also emphasize heredity. Every pedigree in our project contains at

least two affected first degree relatives (siblings, or parent-child), although not all the affected persons' blood samples could be obtained. That means we did not collect simplex trios composed of affected probands and their healthy parents, which might have been the most popular family dataset for classical TDT (56). We infer that, in general, affected individuals with solid family history will have stronger genetic dosage than sporadic cases do, and thus promote detection power of genetic study. Similar concept has been proposed previously (57-60). While performing family-based association study, we use FBAT instead of classical TDT. FBAT is able to use information from all family members, and deal with the problem that more than one affected individuals are analyzed in a family (45).

In conclusion, our linkage and family-based association study in the largest dataset demonstrated CTLA4 is both in linkage to and associated with GD in Chinese Han population in Taiwan. This result and other previous reports in Caucasian suggest CTLA4 gene, or other genes nearby, may be a susceptibility gene of GD with worldwide importance. Further effort is needed to find the exact functional risk allele.

Acknowledgments

We acknowledge all the GD families who graciously agreed to participate in the study. We are indebted to Dr. Pei-Jer Chen and Dr. Ming-Yang Lai for helpful recommendation and technical support. This work was supported by the National Science Council, Taiwan, ROC (91-3112-B-002-003).

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