ความสัมพันธ์ระหว่างยีน HLA-DQA1, HLA-DQB1 และ HLA-DRB1 กับการเกิดโรค Graves ในประชากรไทย

นางสาวธิดาทิพย์ วงศ์สุรวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546
ISBN 974-17-5626-7

THE ASSOCIATION BETWEEN HLA-DQA1, HLA-DQB1 AND HLA-DRB1 LOCI WITH GRAVES' DISEASE IN THAI POPULATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Microbiology (Inter-Department)
Graduate School
Chulalongkorn University
Academic Year 2003

ISBN 974-17-5626-7

Thesis Title	The Association Between HLA-DQA1, HLA-DQB1 and HLA-DRB1 Loci with Graves' Disease in Thai population
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ธิดาทิพย์ วงศ์สุรวัฒน์ : ความสัมพันธ์ระหว่างยีน HLA-DQA1, HLA-DQB1 และ HLA-DRB1 กับการเกิดโรค Graves ในประชากรไทย (THE ASSOCIATION BETWEEN HLA-DQA1, HLA-DQB1 AND HLA-DRB1 LOCI WITH GRAVES' DISEASE IN THAI POPULATION) อาจารย์ที่ปรึกษา : รศ.พญ. ปรียาจิต เจริญวงศ์; อาจารย์ที่ปรึกษาร่วม: ผศ.พญ.ดร. ณัฏฐิยา หิรัญกาญจน์ ; 111 หน้า ISBN 974-17-5626-7

ยีน human leukocyte antigen (HLA) คือหนึ่งในยีนที่เป็นปัจจัยสำคัญอย่างยิ่งต่อการเกิดโรคภูมิต้านเนื้อเยื่อตัวเองหลายๆ โรคซึ่งร่วมถึงโรคเกรฟ กลไกที่ทำให้ HLA มีความสัมพันธ์อย่างจำเพาะนั้น อาจเป็นเพราะบทบาทหน้าที่ของ HLA ในการนำเสนอ autoantigen หรือความสามารถในการคัดเลือก autoreactive T cell การกระจายของ HLA-DRB1, DQA1 และ DQB1 ถูกวิเคราะห์ในผู้ป่วยโรคเกรฟเทียบกับคนปกติ เพื่อบอกถึงรูปแบบยีนที่มีความสัมพันธ์กับผู้ป่วยเกรฟชาวไทย งานวิจัยนี้จึงใช้วิธี population-base case-control โดยรวบรวมผู้ป่วย 124 คนและคนปกติ 124 คน ซึ่งมีเชื้อสายและถิ่นกำเนิดเดียวกัน ใช้เทคนิค PCR-SSP ร่วมกับ PCR-SSOP เมื่อเปรียบเทียบวิเคราะห์ความแตกต่างของทั้ง 2 กลุ่มพบว่า รูปแบบยีน DRB1*1602 (P = 0.0063; OR = 2.91), DQA1*0102 (P = 0.0028; OR = 1.91) และ DQB1* 0502 (P = 0.0074; OR = 1.94) พบในผู้ป่วยมากกว่าคนปกติอย่างมีนัยสำคัญทางสถิติ เมื่อศึกษา haplotype พบว่า haplotype DRB1*1602-DQA1*0102-DQB1*0502 เพิ่มขึ้นในผู้ป่วยอย่างมีนัยสำคัญเช่นกัน (P = 0.0209; OR = 2.55) นอกจากนี้ยังพบความสัมพันธ์แบบ protection คือพบรูปแบบยีนที่เป็น DRB1*07 และ DQA1*0201 ในผู้ป่วยน้อยกว่าคนปกติอย่างมีนัยสำคัญในค่า OR ที่เท่ากันคือ 0.25 (P = 0.0065) ในขณะที่รูปแบบยีน DQA1*0601 ถูกพบในผู้ป่วยน้อยกว่าคนปกติที่ค่า OR = 0.36 (P = 0.0094) เมื่อวิเคราะห์ haplotype พบ 2 haplotype ที่เป็น protective association คือ DRB1*07-DQA1*0201-DQB1*0201 (P = 0.039; OR = 0.32) และ HLA-DRB1*12-DQA1*0601-DQB1*0301 (P = 0.0025; OR = 0.28) การศึกษาครั้งนี้แสดงให้เห็นถึงความสัมพันธ์ที่ยังไม่มีรายงานในชนชาติอื่นๆ ของ DRB1*1602-DQA1*0102-DQB1*0502 และ HLA-DRB1*12-DQA1*0601-DQB1*0301 กับโรคเกรฟในประชากรไทย ซึ่งนำไปใช้เป็นเครื่องหมายสำหรับยีนที่กำหนดความเสี่ยงในการเกิดโรคเกรฟในคนไทยได้

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4489076020 : MAJOR MEDICAL MICROBIOLOGY
KEYWORD : GRAVES' DISEASE / HLA POLYMORPHISMS/ DQA1/ DQB1/ DRB1 THIDATHIP WONGSURAWAT: THE ASSOCIATION BETWEEN HLA-DQA1, HLA-DQB1 AND HLA-DRB1 LOCI WITH GRAVES' DISEASE IN THAI POPULATION. THESIS ADVISOR : ASSOCIATE PROFESSOR PREEYACHIT CHAREONWONGSE, MD. ; THESIS CO- ADVISOR : ASSISTANT PROFESSOR NATTIYA HIRANKARN, MD. Ph.D. 111 pp. ISBN 974-17-5626-7

Human leukocyte antigen (HLA) genes are one of the most important genetic risk factors for susceptibility to various autoimmune diseases including Graves' disease (GD). The molecular mechanism of specific HLA association might be due to its role in autoantigen presentation or the ability to select autoreactive T cells. The distribution of HLA DRB1, DQA1 and DQB1 alleles were analyzed in GD patients compared to normal control in order to identify the alleles associated with GD in Thai population. Population-base case-control study was designed using 124 unrelated Thai GD patients and 124 healthy unrelated Thai individuals with similar ethnic and geographic background. Molecular HLA typings were performed using PCR-SSP (sequence specific primers) and PCR-SSOP (sequence specific oligonucleotide probes). The significance of differences between the two groups were analyzed by the chi-square test. The allele frequency of subjects positive for HLA-DRB1*1602 (P = 0.0063; OR = 2.91), DQA1*0102 (P = 0.0028; OR = 1.91) and DQB1* 0502 (P = 0.0074; OR = 1.94) alleles were significantly increased among GD patients. The analysis of DRB1*1602-DQA1*0102-DQB1*0502 haplotype demonstrated that it was also significantly increased in GD patients (P = 0.0209; OR = 2.55). In addition, we found that DRB1*07 and DQA1*0201 alleles were negatively associated with similar OR of 0.25 (P = 0.0065) and DQA1*0601 allele was another protective allele with OR of 0.36 (P = 0.0094). Haplotype analysis demonstrated 2 protective haplotypes. DRB1*07-DQA1*0201-DQB1*0201 haplotype (P = 0.039; OR = 0.32) and HLA-DRB1*12-DQA1*0601-DQB1*0301 haplotype (P = 0.0025; OR = 0.28) was significantly decreased in GD patients. This findings of a significant associated of DRB1*1602-DQA1*0102-DQB1*0502 and HLA-DRB1*12-DQA1*0601-DQB1*0301 alleles and haplotypes in Thai with GD has not been documented in any ethnic group. DRB1*1602-DQA1*0102-DQB1*0502 alleles and haplotype is the marker for genetic susceptibility to GD in Thai population.

Field of study Medical Microbiology
Academic year 2003

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ACKNOWLEDGEMENTS

The author wished to express her deepest gratitude to her advisor, Associate Professor Preeyachit Chareonwongse, M.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University, and Assistant Professor Nattiya Hirankarn, M.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University, her co-advisor for their kind excellent supervision and invaluable advice, indispensable help, constructive criticism, guidance throughout the period of the study.

I would like to thank the committee of Medical Microbiology Program for giving me permission to commence this thesis in the first instance, to do the necessary research work. I also would like to thank to Dr.Thiti Snabboon, Department of Medicine, Faculty of Medicine, Chulalongkorn University, for his kindness and help in collecting the GD patients.

I would like to thank to Mrs. Ratchada Inwattana for her kindness in guidance throughout the laboratories and providing the beneficial instrument for this study. I am grateful to Miss.Ingorn Kimkong and Mr. Jeerawat Nakkuntod for helping in laboratories. If I lacked them, this work would not be accomplished.

I am indebted to my external examiner, Dr.Wallaya Jongjaroenprasert, Division of Endocrinology and Metabolism Department of Medicine, Ramathibodi Hospital Faculty of Medicine, Mahidol University for her useful suggestions and comments for this study.

Finally, I would like to express my deepest gratitude to my uncle and my aunt for their love, supporting, encouragement and understanding.

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ABBREVIATIONS

Bp	base pair
CD	cluster of differentiation
CTLA-4	cytotoxic T-lymphocyte antigen 4
95% CI	95% Confidence Interval
°C	degree Celsius
et al.	et alii
GD	Graves' disease
Ig	Immunoglobulin
IL	interleukin
IFN	interferon
kDa	Kilodalton
LMP	Low molecular weight polypeptide genes
HLA	human leukocyte antigen
HSP	heat shock protein
MHC	Major Histocompatibility Complex
μl	microliter
μg	microgram
ml	milliliter
mM	millimolar
MW	molecular weight
ng	nanogram
OR	Odd Ratio
PCR	polymerase chain reaction
SSOP	sequence specific oligonucleotide probe
SSP	sequence specific primer
SDS	sodium dodecyl sulfate

SNP	single nucleotide polymorphism
TNF	tumor necrosis factor
TSH	thyroid-stimulating hormone

CHAPTER I

INTRODUCTION

Graves' disease (GD) or Basedow's disease is an autoimmune thyroid disease characterized by autoantibodies to thyroid stimulating hormone (TSH) receptor. The disease is the most frequent cause of hyperthyroidism. The disorder is one of the most widespread autoimmune diseases and affects up to 0.5% to 1% of the world population, with a strong female-to-male preponderance (5-10 : 1) (Vanderpump and Tumbridge 1999). The etiology is not completely elucidated, but there are several lines of evidence suggesting multifactorial mechanism. The environmental, endogenous and genetic factors are involved in its pathogenesis. The environmental and endogenous determinants remain unknown, stress (Winsa, Adami et al. 1991; Chiovato and Pinchera 1996), sex , iodine (Stanbury, Ermans et al. 1998), vitamin D (Ban, Taniyama et al. 2000; Pani, Regulla et al. 2002) and although it is recognized that cigarette smoking may influence the development of goiter and thyroid-associated ophthalmopathy, and that retroviruses may be involved in the initiation of the disease process in GD (Jaspan, Sullivan et al. 1996). The largest study assessing heredity in GD indicated that more than 40% of patients had a family history of thyroid disease (Bartels 1941). Concordance rates for GD in identical (monozygotic) twins have been reported to be as high as 30–40%, compared with 3-9% in non-identical (dizygotic) twins (Stenszky, Kozma et al. 1985). As identical twins share all of the same genes and non-identical twins share, on average, half their genes, the difference in concordance rates can be explained by genetic factors. More recent work with Danish twins confirmed the importance of genetic factors in the etiology of GD (Brix, Kyvik et al. 1998). These data indicate the involvement of genetic and environment factors in GD.

Two main approaches have been used in the search for susceptibility loci: population-based case–control studies and classical linkage analysis (Gough 2000). Case–control studies are a sensitive method of gene detection and the collection of

subjects is resource-efficient. They require prior knowledge of a candidate gene and are prone to inconsistent results due to false positives that may arise from population mismatch. Linkage analysis is a powerful tool for detecting 'major' genes that does not require a candidate gene and is, therefore, a means of genome screening. This method, however, has limited power to detect genes of intermediate or low effect, and the collection of sibpairs and multiple family members may be difficult. GD has been believed that many genes may be involved. There are some groups of gene present in intermediate or low effect, so linkage analysis is not the useful method to find those susceptibility genes in GD.

The candidate genes of GD have been speculated in immune response genes (e.g. HLA, CTLA-4 and cytokines genes). Many studies have been reported of the association between susceptibility genes and disease. Particularly the association with human leukocyte antigen (HLA) class II genes, has been observed in several ethnic groups. The explanation of these associations is still unclear. The major characteristic of autoimmune thyroid disease is thyroid autoantibody production. Thus, associations between GD and HLA are potentially more meaningful for class II molecules than class I molecules. The HLA molecules encoded by genes within the HLA gene region display an impressive degree of polymorphism. The fundamental role of class II molecules is to present peptide to interact with T cell receptor on $CD4^+$ T cell which is important in present self or non self peptide to T cell and positive and negative selection in T cell development. One hypothesis suggests that the Class II heterodimers encoded by specific HLA alleles have distinct abilities to present autoantigens to the T cell receptor in a manner leading to an aberrant immune response HLA and disease. Another possibility is that there may be another disease susceptibility gene which linked to HLA class II genes.

From the studies in Caucasian populations, there is increasing evidence supporting an association between GD and HLA-DR3 (Mangklabruks, Cox et al. 1991; Badenhoop, Schwarz et al. 1992; Badenhoop, Walfish et al. 1995; Cuddihy and Bahn 1996). Caucasian patients with GD usually present the HLA-DRB1*03 allele in strong

linkage disequilibrium with the HLA-DQB1*0201 and DQA1*0501 alleles (Heward, Allahabadia et al. 1998). In other populations, GD has been associated with the HLA-A2 and HLA-DPB1*0501 in Japanese (Dong, Kimura et al. 1992), HLA-B46, HLA-DR9 and HLA-DQB1*0303 in male Hong Kong Chinese (Cavan, Penny et al. 1994; Wong, Cheng et al. 1999), HLA-A10, HLA-B8, HLA-DQw2 in Asian Indians (Tandon, Mehra et al. 1990), and HLA-DR3, HLA-DR1 in native South Africans (Omar, Hammond et al. 1990). Recently, the role of the HLA-DQA1*0501 allele in the susceptibility to GD has being increasingly reported as having an independent effect on the susceptibility to the disease in patients of distinct ethnic background (Gough 2000); (Barlow, Wheatcroft et al. 1996). These data show the difference of HLA susceptibility or protective alleles in populations of distinct ethnic background. Thus, in this study, HLA class II alleles were used as polymorphic markers by candidate gene approach. Population-based case–control studies were used to identity susceptibility alleles in Thai with GD patients. HLA class II allele analysis was determined by the PCR–sequence specific primer (PCR-SSP), with primers specific for exon 2 HLA-DQA1 and HLA-DQB1 genes, and PCR–sequence specific oligonucleotide probes (PCR-SSOP) for HLA-DRB1 genes. Then analysis of HLA class II allele between GD patient group compare to control group was done by Chi-square.

We hypothesized that the finding of HLA-DQA1, HLA-DQB1 and HLA-DRB1 specific for GD patients would discover the HLA allele and/or haplotype which were GD susceptibility gene marker, and might lead to better understanding of mechanism of GD in Thai population. In addition, our study will provide us with the basic knowledge of the diversity of HLA-DRB1, HLA-DQA1 and HLA-DQB1 in Thai population.

CHAPTER II

OBJECTIVE

The objective of this study was:

To analyze the association between HLA-DQA1, HLA-DQB1 and HLA-DRB1 genes and GD by allele analysis in Graves patients and control group by PCR- SSOP and PCR-SSP

CHAPTER III

LITERATURE REVIEW

Graves' disease (GD)

Graves' disease (GD) is an organ-specific autoimmune disease of the thyroid gland characterized by hyperthyroidism, diffuse goitre, ophthalmopathy. The disease is mediated by autoantibodies that bind to the thyroid-stimulating hormone (TSH) receptor and stimulate thyroid hormone production. These stimulatory Abs belong predominantly to the IgG class and act as TSH agonists (Dogan, Vasu et al. 2003) .

Pathogenesis

GD shares many immunologic features with autoimmune hypothyroidism, including high serum concentrations of antibodies against thyroglobulin, thyroid peroxidase, and possibly the sodium–iodide cotransporter in thyroid tissue (Weetman and DeGroot 1999). The serum concentrations of these antibodies vary among patients, and the antibodies themselves may modify the stimulatory effects of thyroid stimulating antibodies. The thyroid-stimulating antibodies cause not only thyroid hypersecretion but also hypertrophy and hyperplasia of the thyroid follicles, which have a columnar and folded epithelium and little colloid (LiVolsi 2000). The result is the characteristic diffuse goiter (Fig. 1A). Lymphocytic infiltration is often present, occasionally resulting in the formation of germinal centers. These intrathyroidal lymphocytes are a major source of autoantibodies, with contributions from the cervical lymph nodes and bone marrow (Weetman, McGregor et al. 1984) Antithyroid drugs ameliorate the histologic changes (Young, Sherwood et al. 1976).

Autoimmunity to the Thyrotropin Receptor

The mechanism by which thyroid-stimulating antibodies bind to and activate the thyrotropin receptor is not known, (McLachlan and Rapoport 1996) but studies with mutated receptors and thyrotropin-receptor sequences have revealed that thyroid-stimulating antibodies bind to conformational epitopes in the extracellular domain of the thyrotropin receptor. These epitopes make up discontinuous segments that overlap the binding site for thyrotropin (Chazenbalk, Wang et al. 1999); (Kosugi, Ban et al. 1993). The production of thyroid-stimulating antibodies is dependent on T cells, and circulating T cells recognize multiple epitopes of the thyrotropin receptor (Martin, Nakashima et al. 1997). Although thyroid-stimulating antibodies cause Graves' hyperthyroidism, the serum antibody concentrations are very low (Chazenbalk, Wang et al. 1999) and are even undetectable in a few patients. The most likely reason for this finding is assay insensitivity, exclusively intrathyroidal production of the antibodies, or misdiagnosis. The hyperthyroidism and goiter are caused primarily by the ability of the thyroid-stimulating antibodies to increase the production of intracellular cyclic AMP. Some of these antibodies also activate phospholipase A (Rapoport, Chazenbalk et al. 1998), and antibodies with this activity may be especially goitrogenic (Di Paola, Menzaghi et al. 1997).

The Role of Thyroid Cells

In GD, thyroid cells not only are sources of thyroid antigens and the target of thyroid-stimulating antibodies, but also express several molecules that modulate intrathyroidal autoimmunity. In response to interferon-γ produced by infiltrating T cells, the thyroid cells express HLA class II molecules, allowing the cells to present antigens such as the thyrotropin receptor to activated T cells (Bottazzo, Pujol-Borrell et al. 1983). Naïve T cells, which require a costimulatory signal from antigen-presenting cells, do not respond to thyroid cells presenting antigen, (Marelli-Berg, Weetman et al. 1997) because the cells do not have the most important costimulatory molecules, CD80 and CD86, which stimulate T cells by means of CD28. Therefore, the initiation of GD is likely

to involve dendritic cells and B cells that express CD80 and CD86. Later, the presentation of antigen by thyroid cells may exacerbate the autoimmune process, as may the expression of other molecules by thyroid cells, such as CD40, CD54, and interleukin-1 and interleukin-6 (Weetman and DeGroot 1999).

Ophthalmopathy and Dermopathy

Graves' ophthalmopathy is characterized by edema and inflammation of the extraocular muscles and an increase in orbital connective tissue and fat. (Heufelder 1995). The edema is due to the hydrophilic action of glycosaminoglycans secreted by fibroblasts. The inflammation is due to infiltration of the extraocular muscles and orbital connective tissue by lymphocytes and macrophages. The increase in the volume of retrobulbar tissue is responsible for most of the clinical manifestations of ophthalmopathy. The muscle cells are normal until the late stages of ophthalmopathy, when they may become atrophic or fibrotic. The muscle cells of the eyelid are hypertrophic but have little lymphocytic infiltration (Small 1989). Dermopathy is characterized by lymphocytic infiltration of the dermis, the accumulation of glycosaminoglycans, and edema.

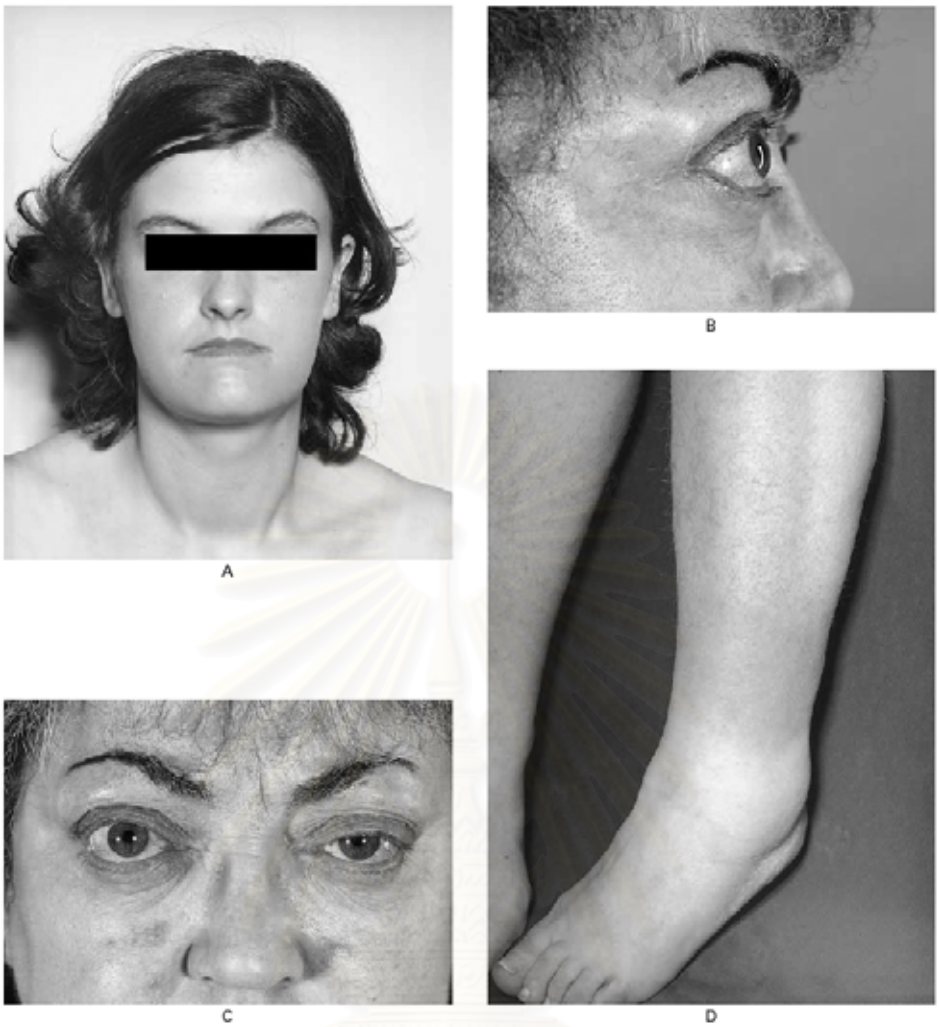


Figure 1 Clinical Manifestations of GD

Panel A shows diffuse goiter in a 28-year-old woman with Graves 'hyperthyroidism.

Panels B and C show ophthalmopathy in a 55-year-old woman with GD, with periorbital edema,chemosis,scleral injection,and proptosis;the lid retraction in this patient is obscured by periorbital edema.

Panel D shows localized dermopathy occurring as an indurated,noninflamed plaque on the anterolateral aspect of the shin of a 47 year old woman.

Epidemiology

Among patients with hyperthyroidism, 60 to 80 percent have GD, depending on regional factors, especially iodine intake. The annual incidence in women over a 20 - year period is around 0.5 per 1000, (Vanderpump and Tumbridge 1999) with the highest risk of onset between the ages of 40 and 60 years; it is thus the most prevalent autoimmune disorder in the United States (Jacobson, Gange et al. 1997). GD is 1/5 to 1/10 as common in men as in women and is unusual in children. The prevalence of GD is similar among whites and Asians, and it is lower among blacks (Vanderpump and Tumbridge 1999).

Clinical Manifestation

The clinical manifestations of GD can be divided into those common to any form of hyperthyroidism and those specific to GD (Table1). The severity and duration of GD and the age of the patient determine the manifestations of hyperthyroidism. The most common symptoms are nervousness, fatigue, a rapid heartbeat or palpitations, heat intolerance, and weight loss; these symptoms are present in more than half of all patients who have the disease. With increasing age, weight loss and decreased appetite become more common, whereas irritability and heat intolerance are less common (Nordyke, Gilbert et al. 1988). Approximately 90 percent of patients who are younger than 50 years old have a firm, diffuse goiter of variable size (Fig. 1A), as compared with about 75 percent of older patients (Nordyke, Gilbert et al. 1988). Glucose intolerance and, rarely, diabetes mellitus may accompany hyperthyroidism. Among patients who are treated with insulin for diabetes, hyperthyroidism increases the insulin requirement. Clinically evident ophthalmopathy (Fig. 1B and 1C) occurs in about 50 percent of patients, in 75 percent of whom the eye signs appear within a year before or after the diagnosis of hyperthyroidism. However, imaging studies reveal evidence of ophthalmopathy, in the form of enlarged extraocular muscles, in most patients without clinical signs (Villadolid, Yokoyama et al. 1995). Older men are at highest risk of severe ophthalmopathy (Perros, Crombie et al. 1993). The prevalence of clinically evident

ophthalmopathy is lower in Asians than in whites (Tellez, Cooper et al. 1992). About 90 percent of patients with ophthalmopathy have hyperthyroidism; the remainder have autoimmune hypothyroidism or are euthyroid at presentation. The most frequent signs of ophthalmopathy (Table 1) are eyelid retraction or lag and periorbital edema. Exophthalmos (proptosis) occurs in up to a third of patients, and diplopia occurs in 5 to 10 percent. Compression of the optic nerve at the apex of the orbit may cause visual loss but is rare. Localized dermopathy is most frequent over the anterolateral aspects of the shin (Fig. 1D), but it can occur at other sites, especially after trauma (Fatourechi, Pajouhi et al. 1994). Dermopathy occurs in 1 to 2 percent of patients with GD, almost always in the presence of severe ophthalmopathy.

Table 1 Major symptoms and signs of hyperthyroidism and of GD and conditions associates with GD (Weetman 2000)

Manifestations of hyperthyroidism	
Symptoms	Signs
Hyperactivity, irritability, altered mood, insomnia	Sinus tachycardia, atrial fibrillation
Heat intolerance, increased sweating	Fine tremor, hyperkinesis, hyperreflexia
Palpitations	Warm, moist skin
Fatigue, weakness	Palmar erythema, onycholysis
Dyspnea	Hair loss
Weight loss with increased appetite	Muscle weakness and wasting
Pruritus	Congestive (high-output) heart failure, chorea, periodic paralysis (primarily in Asian men)
Increased stool frequency	
Thirst and polyuria	
Oligomenorrhea or amenorrhea, loss of libido	
Manifestations of GD	Conditions associated with GD
Diffuse goiter	Type 1 diabetes mellitus
Ophthalmopathy	Addison's disease
A feeling of grittiness and discomfort in the eye	Vitiligo
Retrobulbar pressure or pain	Pernicious anemia
Eyelid lag or retraction	Alopecia areata
Periorbital edema, chemosis, scleral injection	Myasthenia gravis
Exophthalmos (proptosis)	Celiac disease
Extraocular-muscle dysfunction	Other autoimmune disorders associated with the HLA-DR3 haplotype
Exposure keratitis	
Optic neuropathy	
Localized dermopathy	
Lymphoid hyperplasia	
Thyroid acropachy	

Etiology

Susceptibility to GD is determined by a mixture of environmental, endogenous and genetic factors, which are responsible for the emergence of autoreactivity of T and B cells to the thyrotropin receptor. The mechanisms involved are unknown.

Environmental and endogenous factors

Gender : Perhaps the clearest association with autoimmune disease is being a member of the female sex, which carries a 10 fold risk compared to the male sex. Despite this obvious association, the mechanism has remained obscure. The association carries through not only for autoimmune thyroid disease but also for the development of multinodular goiter, and even differentiated thyroid carcinoma, but not undifferentiated thyroid carcinoma. Thus female gender may endow a generally greater reactivity of the thyroid gland, or may subject it to greater stress in some manner. It has been suggested that there may be specific receptors on the promoter for DR genes, which makes them responsive to the estrogen receptor.

Other Suggested Causative Factors : A variety of other ideas have been presented as the cause of GD, but remain unlikely or unproven. Mutation of T or B cells to produce a specific reactive clone has been suggested (Volpe, Farid et al. 1974). Somatic mutation is a known part of the development of B cell clones, whereas specific mutations of B cells or T cells can produce tumors rather than a disease producing autoimmunity. The idea that there could be a deficiency of suppressor cells has been much reported, and there is considerable evidence that something like this is true in autoimmune thyroid disease (Aoki, Pinnamaneni et al. 1979; Sridama, Pacini et al. 1982).

Stress : A recent study in Yugoslavia indicated that patients with GD had suffered on average more stressful episodes than control subjects, but previous similar studies have failed to show this relationship (Winsa, Adami et al. 1991; Chiovato and Pinchera 1996). Stress induces a variety of physiologic responses including anxiety,

tachycardia, restlessness, etc., which are not unlike symptoms of GD. Its role remains enigmatic in causation of GD to this date. A mechanistic route from stress to the development of GD is not obvious. Theoretically, stress might cause activation of the adrenal cortex or the sympathetic nervous system.

Excess Iodide : Iodide itself has been thought to induce GD. This syndrome refers to the occurrence of thyrotoxicosis following supplementation of iodide in medicinals or by salt iodinization. Excess iodide clearly does induce hyperthyroidism in patients with multinodular goiter (Stanbury, Ermans et al. 1998).

Possibly increased iodide intake can actually augment thyroid autoimmunity through other mechanisms. For example, increased iodide intake has been correlated with an increase in incidence of AITD (Boukis, Koutras et al. 1983). This could in theory work by augmenting iodination of TG, and heavily iodinated TG is more immunogenic in animals than is poorly iodinated TG. Also, under special circumstances excess iodide can induce thyroid cell necrosis, and this might liberate antigens.

Whether an excess of iodide can induce true GD and autoimmunity remains unknown. Iodide is one of the most rapid acting agents in suppressing thyrotoxicosis. While it has this effect in most individuals with GD, its action tends to be partial or transient, and thus is not relied upon as an effective antithyroid agent. A recent study suggests that the ability of iodide to suppress GD may be because iodide down-regulates MHC Class I and II expression on thyroid cells (Ingbar and Freinkel 1958).

Smoking has been related to GD, and more specifically to a greater propensity to develop ophthalmopathy, or to have worsening of the condition (Bartalena, Bogazzi et al. 1995).

Genetic Factors

Evidence for genetic factors may be seen in the clustering of these diseases within families and in twin studies. The largest study assessing heredity in GD indicated that more than 40% of patients had a family history of thyroid disease (Bartels 1941). This conditions are frequently found in members of the same family, supporting the genetic hypothesis and implying a shared genetic predisposition. Twin studies provide evidence for both a genetic and an environmental contribution to disease. Concordance rates for GD in identical (monozygotic) twins have been reported to be as high as 30–40%, compared with 3-9% in non-identical (dizygotic) twins (Stenszky, Kozma et al. 1985).. As identical twins share all of the same genes and non-identical twins share, on average, half their genes, the difference in concordance rates can be explained by genetic factors. More recent work with Danish twins (Brix, Kyvik et al. 1998) confirmed the importance of genetic factors in the aetiology of GD. The lower pairwise concordance rates, however, imply that the genetic effect might not be as strong as originally believed. In all epidemiological studies, concordance rates for identical twins are substantially less than 100%, providing evidence that other environmental factors play an aetiological role.

GD is likely to be polygenic disorders with several genetic regions, termed susceptibility loci, contributing to inheritance. Such ‘genes’ are neither sufficient alone nor necessary to cause disease (Heward and Gough 1997). The search for susceptibility loci is ongoing, with the aims of gaining a better understanding of disease pathophysiology and of being able to predict outcome of disease at onset and to identify other family members at risk of disease, all of which will improve disease management. Three main strategies have been applied in humans to determine the identity of susceptibility loci. These include population-based case–control studies, classical linkage analysis, and intrafamilial linkage disequilibrium.

Study Approaches

Three main approaches have been used in the search for susceptibility loci: population-based case–control studies, classical linkage analysis, and intrafamilial linkage disequilibrium.

Linkage analysis

The approach of linkage analysis in groups of affected sibpairs or multiplex families, is a powerful tool for detecting major genes in complex diseases, particularly as no candidate is required. This approach has been used successfully in type 1 diabetes, with a genome-wide search identifying at least 14 susceptibility loci (Davies, Kawaguchi et al. 1994). Linkage analysis has been performed at various regions of interest, including the HLA region on chromosome 6p (Roman, Greenberg et al. 1992) (Ratanachaiyavong and McGregor 1994) (Shields, Ratanachaiyavong et al. 1994), the thyrotrophin receptor gene (TSH-R) on chromosome 14q (de Roux, Shields et al. 1996) and GD-1 on 14q31 (Tomer and Davies 1997) (Table 2). And other important genetic susceptibility loci with high lod score have been identified on chromosomes 5q31 (Jin, Teng et al. 2003), Xp11 (Imrie, Vaidya et al. 2001), 20q11-2 (Tomer, Barbesino et al. 1998; Pearce, Vaidya et al. 1999), 18q21 (Vaidya, Imrie et al. 2000), 2p21 (Maalej, Makni et al. 2001), 2q33 (Vaidya, Imrie et al. 1999). Studies of the HLA region have been performed in GD (Roman, Greenberg et al. 1992), but have failed to detect linkage. Similar results have been obtained for the CTLA-4 gene (Barbesino, Tomer et al. 1998) and the TSH-R gene (de Roux, Shields et al. 1996). Although authors claim to have data sets of sufficient size to detect linkage, positive results for regions such as HLA have not been found and they suggest, therefore, that this region can be exerting only a modest effect on disease. Loci including the HLA region may well be exerting a modest effect on disease but, as the total genetic contribution to GD may also be modest, such regions are likely to be major contributors to overall genetic susceptibility.

In summary, the approach of linkage analysis is a powerful tool for detecting major genes in complex diseases. This method has limited power to detect genes of modest effect and it is difficult to collect samples from families that include both parents.

Table 2 Previously reported susceptibility loci with evidence of linkage to GD

Chromo-some	Locus	Marker	Pheno-type	Type of study	Type of analysis	Results	Ref.
2	2q33 (CTLA-4)	D2S155	TAb	WGS	Ped-LOD	HLOD = 4.2	(Tomer, Greenberg et al. 2001)
2	2q33 (CTLA-4)	D2S117	GD	CLA	ASP-NPL	NPL = 3.4	(Vaidya, Imrie et al. 1999)
5	5q31	D5S356	AITD	WGS	ASP-LOD	LOD = 3.1	(Sakai, Shirasawa et al. 2001)
6	6p21 (HLA)	D6S273	GD	CLA	ASP-NPL	NPL = 1.95	(Vaidya, Imrie et al. 1999)
6	6p11 (AITD-1)	D6S257	AITD	WGS	Ped-LOD	LOD = 2.9	(Tomer, Barbesino et al. 1999)
6	6p11	D6S257	HT	WGS	One large pedigree	LOD = 1.52	(Alkhateeb, Stetler et al. 2002)
						NPL = 7.53	
8	8q24 (Tg)	D8S272	AITD	WGS	ASP-LOD	LOD = 2.3	(Sakai, Shirasawa et al. 2001)
8	8q24 (Tg)	D8S284	AITD	WGS	Ped-LOD	HLOD = 3.5	(Tomer, Ban et al. 2003)
12	12q22 (HT-2)	D12S351	HT	WGS	Ped-LOD	HLOD = 2.3	(Tomer, Barbesino et al. 1999)
14	14q31	D14S81	GD	WGS	Ped-LOD	LOD = 2.1	(Tomer, Barbesino et al. 1999)
18	18q21 (IDDM-6)	D18S487	GD	CLA	ASP-NPL	NPL = 3.1	(Vaidya, Imrie et al. 2000)
20	20q11 (GD-2)	D20S195	GD	WGS	Ped-LOD	LOD = 3.5	(Tomer, Barbesino et al. 1999)
20	20q11 (GD-2)	D20S106	GD	CLA	ASP-NPL	NPL = 2.01	(Pearce, Vaidya et al. 1999)
X	Xp11 (IDDMX)	DXS8083	GD	CLA	ASP-NPL	NPL = 2.01	(Imrie, Vaidya et al. 2001)

NPL = nonparametric linkage analysis, LOD = logarithm of odds, CLA = candidate locus analysis, WGS = whole genome screening, ASP = affected sib-pairs, Ped = multiplex multigenerational pedigrees

Population-based case–control studies

Population-based case–control studies investigate association of a marker allele with disease by comparing the frequency of the allele in a diseased population with that in a disease-free population. disease, there will be a significant excess of the marker allele in the disease population. It is not possible, however, to state from such studies whether the associated allele is the primary disease-causing mutation, or whether the allele is acting as a marker and is in linkage disequilibrium with a nearby unknown aetiological mutation. Either way, such studies require prior knowledge of a candidate gene with a known or suspected biological function being implicated in the pathogenesis of the disease. Although widely used, this methodology has met with limited success in identifying susceptibility loci for GD. The association between GD and HLA-B8 and DR3, which has been observed in many studies of whites, has also been observed in South African blacks (Omar, Hammond et al. 1990). However, GD is associated with different class I and II HLA antigens in other ethnic groups, as well as in subdivisions of a major ethnic group (Chinese). The recent study focus on the class II region more than class I region because of function of class II molecule. Several case–control studies have demonstrated association of alleles of the class II HLA region on chromosome 6p with GD (Table 3). In the majority of these studies, results have been consistent, with allelic association reported between GD and the alleles DR3 and DQA1*0501 (Yanagawa, Mangklabruks et al. 1993; Barlow, Wheatcroft et al. 1996; Heward, Allahabadia et al. 1998; Marga, Denisova et al. 2001). Because of the sensitivity of the case–control method and results already reported, most adequately sized, well matched data sets would be expected to identify the HLA region as a susceptibility locus.

Several other loci, including the thyrotrophin receptor gene (Cuddihy, Dutton et al. 1995) (Kotsa, Watson et al. 1997) and interleukin-1 receptor antagonist gene (Blakemore, Watson et al. 1995; Cuddihy and Bahn 1996), have yielded conflicting results in autoimmune thyroid disease. Such inconsistencies highlight the inherent problems with the population-based case–control method. False positive associations

arising as a result of a chance event or random variation are highly likely in small data sets, and many case–control studies have been performed on data sets of inadequate numbers. Moreover, it is almost impossible to obtain perfectly matched diseased and normal control populations. False positives arising from population stratification (Gough, Saker et al. 1995) are also more likely to occur in small data sets. The major problem of population stratification can be overcome by using family-based association studies. Despite their limitations, however, population-based case–control studies do have a number of advantages. First, the identification and collection of samples from subjects is far quicker and more resource efficient than the collection of family samples. Secondly, the population-based case–control study is more sensitive than some of the family-based studies and is more likely to detect genes of modest effect.

Table 3 Positive association between genetic markers in the MHC locus and GD

Association HLA (susceptibility)	Ethnic	N	OR/RR	References
Caucasians				
DR3 (DRB1*03)	Caucasians	130	3.4	(Mangklabruks, Cox et al. 1991)
		374	2.3	(Badenhoop, Schwarz et al. 1992)
		134	3.5	(Cuddihy and Bahn 1996)
DR3 (DRB1*03)	Blacks (South Africa)	nd	nd	(Omar, Hammond et al. 1990)
DQA1*0501	Caucasians	542	2.5	(Badenhoop, Walfish et al. 1995)
DR3 & DQA1*0501	Caucasians	169	2.5&3.7	(Yanagawa, Mangklabruks et al. 1993)
		177	2.7&3.8	(Barlow, Wheatcroft et al. 1996)
		47	3.6&2.35	(Marga, Denisova et al. 2001)
		592	2.7&3.2	(Heward, Allahabadia et al. 1998)
Asians				
DR8	Japanese & Koreans	88,128	1.7,2.3	(Inoue, Sato et al. 1992), (Cho, Rhee et al. 1987)
DR5	Koreans	128	4.4	(Cho, Rhee et al. 1987)
DPB1*0501	Japanese	76	5.32	(Dong, Kimura et al. 1992)
DR9	Hong Kong Chinese	97	2.16	(Cavan, Penny et al. 1994)
DQB1*0303			3.19	
DQB1*0303		67	4.22	(Wong, Cheng et al. 1999)
DR2	Taiwanese	93	2.49	(Tsai, Hsieh et al. 1989)
DR9	Taiwanese and Chinese(male)	93,159	2.69,3.2	(Tsai, Hsieh et al. 1989; Yeo, Chan et al. 1989)
DRB1*0901	Taiwanese	236	1.62	(Huang, Wu et al. 2003)
DRB1*1403, DQA1*0103	Japanese	62	nd	(Katsuren, Awata et al. 1994)

nd = no data

* = not significant

Table 4 Negative association between genetic markers in the MHC locus and GD

Association HLA (resistance)	Ethnic	N	OR/RR	Reference
Caucasians				
DR7	Caucasians	nd	nd	(Mangklabruks, Cox et al. 1991) (Chen, Huang et al. 1999) (Lavard, Madsen et al. 1997)
DR5	Caucasians	nd	nd	(Kraemer, Donadi et al. 1998)
DQA1*0201	Caucasians	nd	nd	(Yanagawa, Mangklabruks et al. 1994)
DQB1*0602	Caucasians	75	0.18	(Maciel, Rodrigues et al. 2001)
Asians				
DRB1*07	Taiwanese	236	nd	(Huang, Wu et al. 2003)
DQB1*0301, DQA1*0401 and DR12	Chinese	nd	nd	(Cavan, Penny et al. 1994)
DQB1*0301	Singaporean Chinese	35	weak	(Chan, Lin et al. 1993)
DQB1*0501	Japanese	67	nd	(Tamai, Kimura et al. 1994)
DQB1*0201	Hong Kong Chinese	67	0.2	(Wong, Cheng et al. 1999)

nd = no data

Intrafamilial linkage disequilibrium

The method of linkage disequilibrium analysis within families is gaining recognition as a powerful alternative to classical linkage analysis in the search for susceptibilty genes in complex disease. The Transmission Disequilibrium Test (TDT) is a test for linkage in the presence of linkage disequilibrium (Spielman, McGinnis et al. 1993) distortion as an alternative explanation to linkage disequilibrium. The TDT may also be used in families with two or more affected offspring. This technique was first used to detect linkage of the insulin gene region in type 1 diabetes (Spielman, McGinnis et al. 1993). More recently, the TDT has confirmed linkage in the presence of linkage disequilibrium for the MHC class II region with GD (Heward, Allahabadia et al. 1998), with alleles previously shown to be associated with GD in various case–control studies (Table 3).

The main limitation of intrafamilial linkage disequilibrium analysis is that it requires knowledge of candidate genes before the test can be performed, and a polymorphism within the gene, or one in strong disequilibrium with it, must be available.

The Major Histocompatibility Complex Region

The human major histocompatibility complex (MHC) region on chromosome 6p21 is called the human leukocyte antigen (HLA) system. The MHC-HLA system has a crucial role not only in the immune response to foreign material but also to self-antigen. It can be subdivided into three main gene clusters. The class I region genes include those encoding the alpha peptide chains of HLA-A, -B, and -C antigens, which are membrane-bound proteins expressed on the cell surface of nucleated cells involved in the presentation of endogenous peptides to cytotoxic ($CD8^+$) T lymphocytes. The class II region genes encode molecules largely expressed on specialized antigen-presenting cells, including macrophages, B lymphocytes, and, under certain circumstances, other cell types. In this way, the class II genes are likely to be involved in the autoimmune process. Class II molecules bind peptides generated by the degradation of proteins in the endocytic pathway. As a result, the class II antigen complex is an important mechanism for stimulating the T-cell receptor on $CD4^+$ and T lymphocytes. The class III region contains many genes encoding immune regulator proteins, including some of the cytokines.

The Genetics of the HLA

The HLA complex is a gene cluster of various loci grouped together on single region. Gene mapping studies have indicated that the HLA occupies 3.5 megabases of DNA on the short arm of chromosome 6 in the distal portion of the 6p21.3 band. The loci of the HLA complex may be divided into three classes based on certain functional characteristics of genes within each class: class I, class II and class III. The organization of the genes that encode HLA molecules is shown in Figure 2 (Simmonds and Gough 2004).

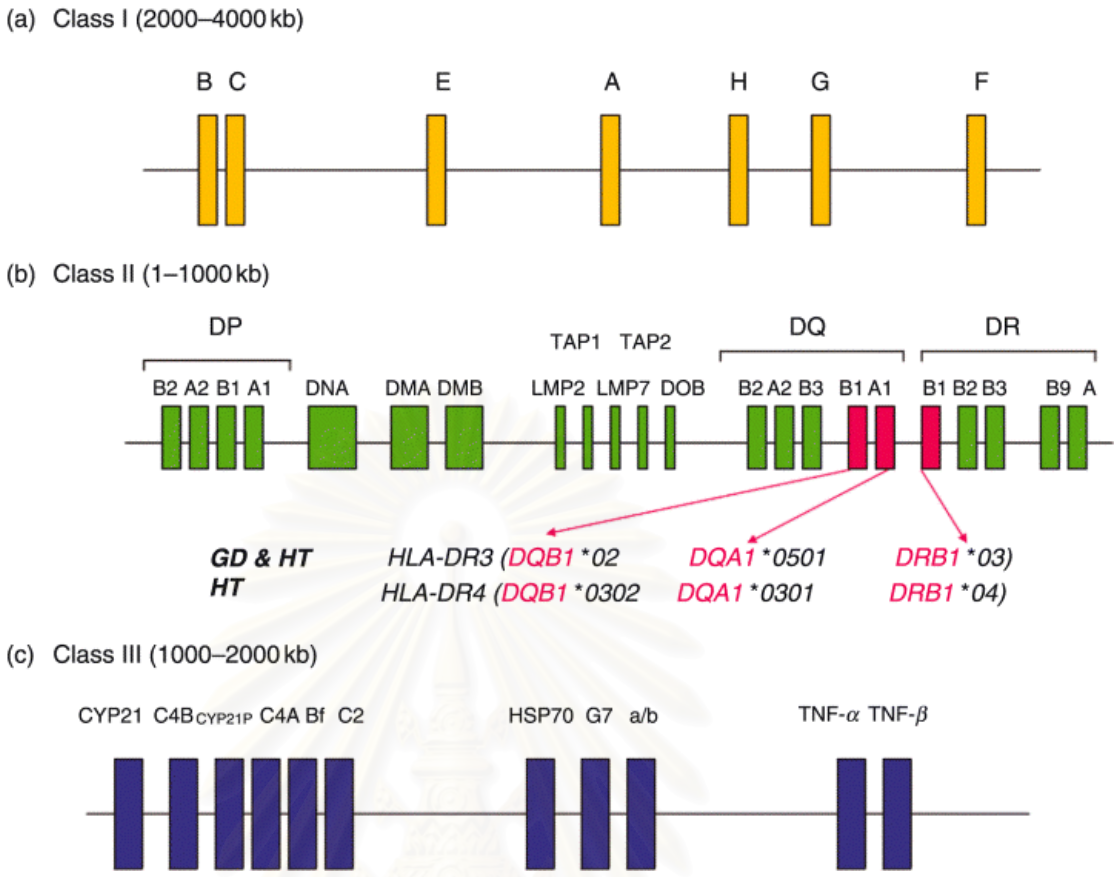


Figure 2. HLA regions (a) HLA class I gene region (b) HLA class II gene region with known haplotypes associated with GD (transport associated with antigen processing genes (*TAP1* and *TAP2*) and large multifunctional protein (*LMP*)). (c) HLA class II gene regions (Heat shock protein (*HSP*), tumour necrosis factor (*TNF*)) (Simmonds and Gough 2004).

1. Class I HLA genes

The class I genes of the HLA complex can divided into 2 types. There are classical HLA class Ia and non-classical HLA class Ib. The classical HLA class Ia contains three loci, called HLA-A, HLA-B and HLA-C loci, spread over a region of 2 Mb. The HLA-E, -F and –G genes encode non-classical HLA class Ib proteins. The class I HLA genes appear to be coordinately controlled with the beta two-microglobulin gene located on chromosome two. Although the beta two-microglobulin gene is not an HLA gene, the gene does code for a subunit associated with the alpha chain of HLA I

molecule. Studies indicate that the expression of the class I molecule is dependent upon the expression of the beta two-microglobulin gene. A class I gene is comprised of eight exons. The first exon encodes a signal peptide that directs the insertion of the HLA I molecule into the endoplasmic reticulum during translation. Exon two through four encodes the three external domains of the protein (alpha 1-3). The fifth exon encodes the transmembrane domain, while exons six through eight encode cytoplasmic domains. Polymorphisms are on exons two and three

2. Class II HLA genes

The genetic locus encoding class II molecules is known as the D region in humans. The D region is further subdivided into DP, DQ, DR, DO and DN (Bell, Denny et al. 1985). The class II genes are made up of both alpha and beta genes. Similar to class I genes, the genomic arrangement of class II genes reflects the domain structure of class II proteins. The first exon of both alpha and beta chain genes encode the 5' untranslated region, the leader or signal sequences. Exons two and three code for either the alpha one and alpha two or the beta one and beta two domains. Exon four of the alpha genes encodes the transmembrane cytoplasmic domain and part of the 3 untranslated regions, while exon four of the beta genes encodes the transmembrane and a portion of the cytoplasmic domain. Exon five encodes the rest of the untranslated region in the alpha genes and the rest of the cytoplasmic domain of the beta genes.

3. Class III HLA genes

The class III HLA genes encode complement components (C2, C4 and factor B) that show no structural similarity to either class I or class II molecules. These genes, along with genes encoding heat-shock protein-70 components and the peptide transporters that function in the loading of HLA class I molecules.

HLA polymorphism

One of most striking features of the HLA is the extensive polymorphism of the class I and II genes and their encoded products. The number of definable allelic products of a given locus in a species has been increased by development of new methodology. The HLA Sequence Database which holds information on HLA sequences currently contains 1,711 allele sequences. In addition to the physical sequences, the database contains detailed information concerning the material from which the sequence was derived and data on the validation of the sequences. To date (March 2004), some 303 HLA-A, 559 HLA-B, 150 HLA-C, 6 HLA-E, 2 HLA-F and 15 HLA-G class I alleles have been named. A total of 3 HLA-DRA, 439 HLA-DRB, 25 HLA-DQA1, 56 HLA-DQB1, 20 HLA-DPA1, 107 HLA-DPB1, 4 HLA-DMA, 6 HLA-DMB, 8 HLA-DOA and 8 HLA-DOB class II sequences have also been assigned (http://www.ebi.ac.uk/imgt/hla/stats.html)

Structure of HLA molecules

There are two main kinds of HLA molecules, class I and class II. Class I molecules are found on all nucleated cells, while class II molecules show a more restricted expression pattern. Class II molecules are always expressed by B lymphocytes, interdigitating dendritic cells, and thymic epithelial cell. Other cells, such as macrophages, activated T lymphocytes and endothelial cells can be induced to express class II molecule by cytokine such as IFNy.. Both kinds of molecules belong to a group of molecules known as the immunoglobulin supergene family, which includes immunoglobulins, T cell receptors, CD4, CD8 and others. The class I and class II are described in this topic.

1. Class I HLA molecules

Class I HLA molecules are composed of a heavy chain non-covalently associated with beta2-microglobulin. The HLA-encoded polypeptide is about 350 amino acids long and glycosylated, giving a total molecular weight of about 45 kDa.

This polypeptide folds into three separate domains called alpha-1, alpha-2 and alpha-3. Beta two microglobulin is a 12 kDa polypeptide that is non-covalently associated with the alpha –3 domain. Between the alpha-1 and alpha-2 domains lies a region bounded by eight antiparallel beta-pleated sheets on the bottom and two alpha helices on the sides. This region is capable of binding (via non-covalent interactions) a small peptide of eight to ten amino acids (Roitt 1996).

2. Class II HLA molecules

Class II HLA molecules are composed of two polypeptide chains, both encoded by the D region. These polypeptide (alpha and beta) are about 230 and 240 amino acids long, respectively, and are glycosylated, giving molecular weights of about 33 kDa and 28 kDa. These polypeptide fold into two separate domains, alpha-1 and alpha-2 for the alpha polypeptide, and beta-1 and beta-2 for the beta polypeptide. Between the alpha-1 and beta-1 domains lies a region very similar to that seen on the class I molecule. This region, bounded by eight beta-pleated sheets on the bottom and two alpha helices on the sides, is capable of binding (via non-covalent interactions) a small peptides of to twenty-four amino acids (Roitt 1996).

A certain amount of redundancy and polymorphism is built into the MHC-restricted response. Heterozygous humans can have at least six different MHC alleles. Each MHC is optimized to bind a different array of peptides, raising the chances that for any complex protein, at least one strong-binding epitope will be presented to helper T cells. Each MHC class II molecule is probably under evolutionary pressure to bind peptides promiscuously, further broadening potential immune responses. Since residues that from hydrogen bonds with the peptide main chain are mostly conserved and somewhat difficult to access experimentally, most studies have focus on the specific anchor-pocket interactions of different MHC class II proteins HLA-DR and HLA-DQ

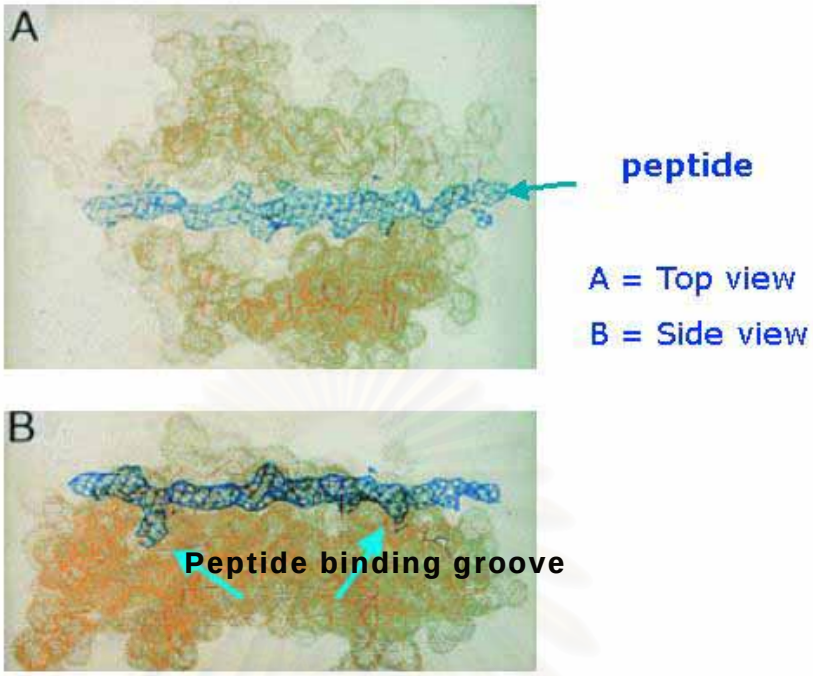


Figure 3 HLA class II molecule a) Top view and B) Side view
(http://cai.md.chula.ac.th/lesson/lesson4503/index.html)

HLA-DR protein

HLA-DR has been heavily investigated both for peptide binding and disease association. The products of DR alleles are classified by α and β chain, which in commonly studied MHC proteins is sometimes condensed to a single number. HLA-DR1 was the first MHC protein whose crystal structure was solved. Structures of other DR allele products were soon solved (e.g.,DR3 and DR7), in addition to structures of DR1 bound to specific peptides. The structures of other DR allele products are highly similar to the original DR1 structure with allele-distinct polymorphisms clustered around the peptide anchor pockets, providing different kinds of pocket specificities.

HLA-DQ protein

This counterpart of DR is of considerable interest because of its disease associations. Certain HLA-DQ alleles are associated with IDDM (Abbas 1997) and celiac disease (Hurley, Nunez et al. 1982), among other. Binding motifs have been proposed for HLA-DQ from directed peptide-truncation and mutation studies (Ettinger and Kwok 1998). Homology modeling starting with the HLA-DR structure has led to models of anchor pockets of some DQ proteins. (van de Wal, Kooy et al. 1997; Ettinger, Liu et al. 2000).

HLA-DQ and DR differ in patterns of polymorphism. Most of the distinguishing polymorphisms for DR are found in its β chain, while the DR α chain is largely invariant (Hurley, Nunez et al. 1982). In contrast, both chains of DQ contain numerous polymorphisms. The implication of this is that DR β chains are often interchangeable in their capability to form heterodimers with the α chain, while allelic forms of DQ α and β chains are usyally paired and not interchangeable.

Nomenclature of HLA alleles

Initially, class II polymorphism was defined serologically using antibodies and by the mixed lymphocyte reaction (the proliferation of T-cells in response to cells from other persons expressing different class II molecules). Now class II polymorphisms have been defined by molecular method, RFLP, direct sequencing, PCR-SSP and PCR-SSOP. Further, it is now clear that the polymorphism is much greater than was detected serologically or by mixed lymphocyte reactions. Consequently, a new nomenclature has been introduced for the HLA system, which provides a way of describing the alleles for individual α- and β- (now renamed A and B) chains (Bodmer, Marsh et al. 1992).

Serological Nomenclature: Defined proteins, not alleles. Antigen families defined by antibody reaction patterns.

Molecular Nomenclature: Types alleles, not proteins. Allele families defined by sequence similarity as influenced by serological reactivities (if known) of expressed proteins.

Table 5 Nomenclature of HLA alleles
{http://www.anthonynolan.org.uk/HIG/lists/nomenlist.html #199}

Nomenclature	Indicates
HLA	the HLA region and prefix for an HLA gene
HLA-DRB1	a particular HLA locus i.e. DRB1
HLA-DRB1*13	a group of alleles which encode the DR13 antigen
HLA-DRB1*1301	a specific HLA allele
HLA-DRB1*1301N	a null allele

HLA and GD

Initial association studies in GD focused on the Class I region, with association of the HLA-A8 region (Grumet, Payne et al. 1974) and in particular the HLA-B8 region (Farid, Barnard et al. 1976) with disease. Further studies demonstrated strong linkage disequilibrium (LD) between HLA-B8 and HLA-DR3 (Bech, Lumholtz et al. 1977) suggesting that the association of HLA-B8 may be secondary to DR3 and that the HLA-DR region played a greater role (Farid, Sampson et al. 1979). Such assumptions were based on the finding of greater degrees of association between disease and DR3 compared with HLA-B8. While such assumptions may be correct a comprehensive statistical analysis has not been performed and class I genes can not therefore be completely excluded from having an independent effect. However, the majority of future studies have focused on the HLA class II region. As has been mentioned earlier, products of the class II gene region aid in antigen presentation to CD4 Th cells. The DRB1, DQA1 and DQB1 genes are highly polymorphic and these molecules play a major role in maintaining tolerance to self-thyroid antigens. With aberrant expression being seen on follicular cells (the target cells for GD) and on activated lymphocytes, the class II genes and molecules are attractive primary candidates for involvement with AITD.

Farid *et a*l. in 1979 (Farid, Sampson et al. 1979) were the first group to use a case control based method to show a strong association with HLA-DR3 in Canadian Caucasians, with several other studies replicating this result (Allanic, Fauchet et al. 1980) (Dahlberg, Holmlund et al. 1981) (Boehm, Kuhnl et al. 1992). Despite the strong association of DR3 with disease in Caucasians it has been observed that DR3 frequency is reduced in Sardinian GD patients (Boehm, Kuhnl et al. 1992) and disease association in the Japanese and Chinese population seemed to be due exclusively to class I molecules (Ito, Tanimoto et al. 1989). These and other studies suggest that either different HLA associations are contributing to disease in different populations or that those loci showing positive association are in LD with an as yet unknown primary disease determinant in the HLA region. A number of studies have subsequently

replicated association between GD and DR3 in both case control and family based datasets and gone on to demonstrate association between the DRB1*03-DQB1*02-DQA1*0501 extended haplotype and GD conferring relative risks (RR) for the development of disease of between 1.9 and 3.8 (Gough 2000). Attempts have been made to statistically split the haplotype to determine whether individual class II genes are conferring susceptibility to disease or whether this really is a haplotype effect. Yanagawa et al. (Yanagawa, Mangklabruks et al. 1993) have reported a significant increase in DQA1*0501 in USA Caucasian subjects with GD and a trend towards association of DR3 with GD, and proposed that the DQA1*0501 association was independent of DR3 with DQA1*0501 conferring a greater risk (Yanagawa, Mangklabruks et al. 1993). Confirmation of stronger independent association of DQA1*0501 with GD was shown by the result still being present in DR3 negative subjects (Barlow, Wheatcroft et al. 1996). However, this study did not correct for the number of alleles seen, which when performed leads to a non significant finding. Other authors have reported association of DQA1*0501 with GD and others have attempted to determine if association of the DQA1*0501 with GD is the result of LD with DR3 (Cuddihy and Bahn 1996; Hunt, Marshall et al. 2001), or whether *DQA* posed a greater, independent risk (Maciel, Rodrigues et al. 2001) (Table 3). Inconsistencies reported in such studies are probably the result of small sample groups and comparisons between differing geographical and ethnic backgrounds.

Heward *et a*l. 1998 (Heward, Allahabadia et al. 1998) performed the largest case control investigation of association of the class II region with GD in a UK population to date, and provided confirmation in an independent family dataset. Strong association of both DRB1*03 (RR =2·45) and DQA1*0501 (RR =2·26) was found but no independent effect of DQA1*0501 was seen. Due to linkage between DRB1*03, DQB1*02 and DQA1*0501, distribution of the haplotype DRB1*03-DQB1*02-DQA1*0501 between GD and control subjects was analysed, showing association with GD (RR =2·52) which was confirmed in the family dataset (Heward, Allahabadia et al. 1998). In summary, therefore, there are consistent associations between the HLA class II genes and GD although at the present time it remains unclear whether the primary susceptibility is the

result of a haplotype or single locus effect. Ultimately, it would be important to know whether polymorphisms leading to specific amino acid changes within peptide binding pockets of HLA class II molecules are determining the nature of antigen presentation and in turn the T cell repertoire conferring susceptibility to GD.

Development of expression of class I or class II HLA molecules on the thyroid epithelial cell was suggested as a factor in the causation of GD by Bottazzo et al (Bottazzo, Pujol-Borrell et al. 1983). It is now apparent that exposure of thyroid epithelial cells to IFN, presumably laborated by infiltrating lymphocytes or other immune cells, can lead to the expression of class II molecules on the thyroid cell surface (Matsunaga, Eguchi et al. 1986). Expression of these molecules does allow the thyroid epithelial cell to function as a weak antigen presenting cell (Eguchi, Otsubo et al. 1988). Class II expression is secondary to the effect of an autoimmune lymphocyte attack and is induced by IFN (Piccinini, Mackenzie et al. 1987). Culture of human thyroid cells from patients with GD *in vitro* shows that class II expression disappears (Mukuta, Arreaza et al. 1997), as it does when the cells are transplanted into nude mice (Leclere, Bene et al. 1984). It is also possible that the class II expression is a defensive response (Markmann, Lo et al. 1988). Antigen presentation to a T cell, in the absence of a second signal, could lead to anergy of the attacking T cell. If the original hypothesis is proven to be correct, it may be that the class II expression is secondary but may play a role in continuing or strengthening the autoimmune reactivity to thyroid antigens.

Kohn et al summarize the model suggests that GD is initiated by an insult to the thyrocyte in an individual with a normal immune system (figure 4). The insult, infectious or otherwise, causes double strand DNA or RNA to enter the cytoplasm of the cell. This causes abnormal expression of major histocompatibility (MHC) class I as a dominant feature, but also aberrant expression of MHC class II, as well as changes in genes or gene products needed for the thyrocyte to become an antigen presenting cell (APC). These include increased expression of proteasome processing proteins (LMP2), transporters of antigen peptides (TAP), invariant chain (Ii), HLA-DM, and the co-stimulatory molecule, B7, as well as STAT and NF-kappaB activation. A critical factor in

these changes is the loss of normal negative regulation of MHC class I, class II, and TSHR gene expression, which is necessary to maintain self-tolerance during the normal changes in gene expression involved in hormonally-increased growth and function of the cell. Self-tolerance to the TSHR is maintained in normals because there is a population of CD8- cells which normally suppresses a population of CD4+ cells that can interact with the TSHR if thyrocytes become APCs. This is a host self-defense mechanism that they hypothesize leads to autoimmune disease (Kohn, Napolitano et al. 2000).

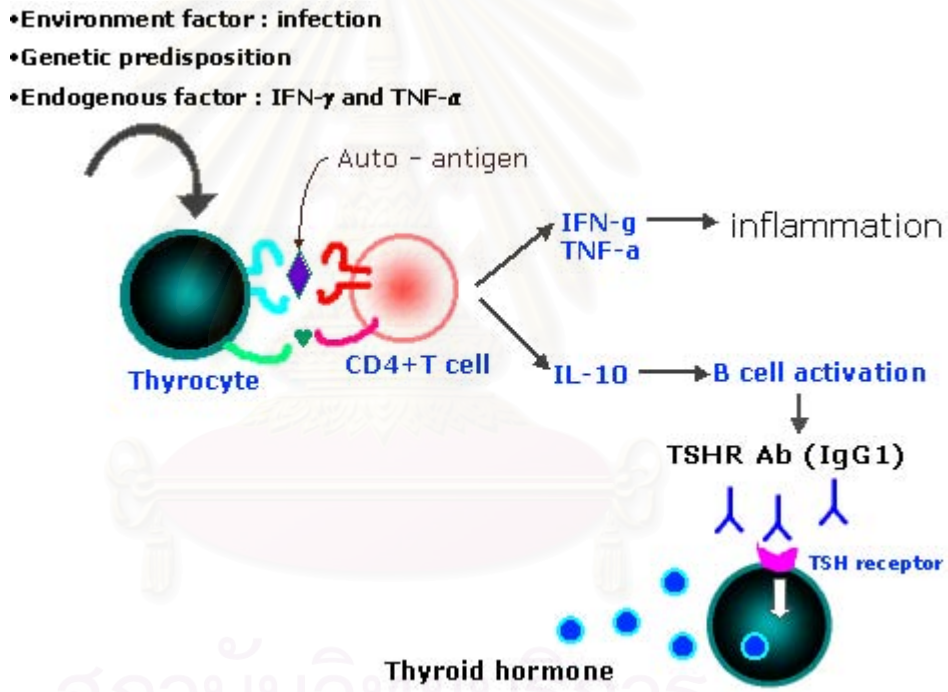


Figure 4 The model suggests to develop GD

Some authors described aberrant HLA-DR expression antigens in follicular thyroid cells in GD (Hanafusa, Pujol-Borrell et al. 1983). On the other hand In Caucasians, GD has repeatedly been associated with HLA-DRB especially HLA-DRB1*03 (Table 3). These can hypothesize in 2 points.

1. HLA-DRB1*03 can bind the thyroid peptide better than other HLA-DR alleles or can not negative select the autoreactive T cells

2. HLA-DRB1*03 is closely linked to susceptibility genes in GD because of linkage disequilibrium with susceptibility gene

The study of Sawai and DeGroot support the first hypothesis. They investigated T cell responses to a battery of synthetic peptides spanning the human TSHR extracellular domain. The results in patients with GD suggested that peptides containing amino acid residues 158–176, 207–222, 237–252/248–263, and 343–362/357–376 of TSHR are important or possibly immunodominant T cell epitopes (Soliman, Kaplan et al. 1995; Fisfalen, Palmer et al. 1997). They use these peptides to examintheinding characteristics to a GD-predisposing HLA class II molecule. DRB1*03 molecules bind TSHR immuonodominant peptide epitopes with intermediate affinity. On the contrary, DR3 binds non-immunogenic peptides either with poor affinity or not at all. These results suggest that susceptibility to GD associated with inheritance of a specific HLA class II gene is due to the influence of the HLA molecule-TSHR peptide complex on the T cell repertoire (Sawai and DeGroot 2000). Either high affinity peptides or extremely low affinity or nonbinding peptides lead to loss of responding T cells due to negative thymic selection or failure of positive selection, respectively. The remaining intermediate to poor affinity peptides could be autoantigenic peptides. This study supports the important role of HLA class II function.

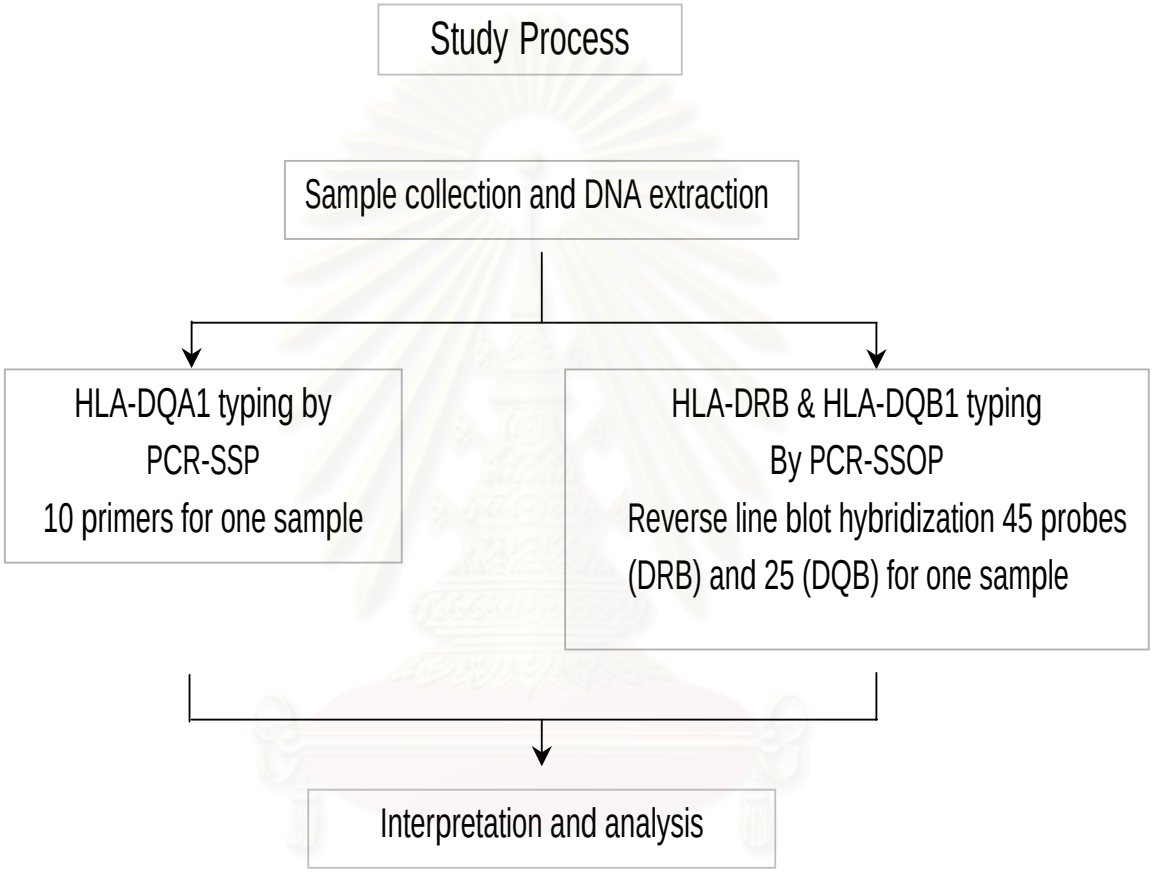
The new recent study of Flynn et al (Flynn, Rao et al. 2004) demonstrate that the DRB1*0301 transgene, expressed in a murine class II-negative NOD strain, was sufficient as a susceptibility allele for a GD model. This NOD strain lacks endogenous class II molecules and cannot respond to mouse or human Tg immunization in the absence of the DR3 transgene. In the presence of DR3, about 30% of mice developed

Graves'-like hyperthyroidism or some sign of the disorder at week 14 after immunization with human TSHR plasmid DNA. Interestingly, lymphocytic infiltration with thyroid damage and Abs to mouse thyroglobulin were also noted. Vector controls were uniformly negative. Thus, DR3 transgenic mice can serve as a model for GD. This study also supports the first hypothesis.

Retrobulbar fibroblasts are the main target of the immune process in Graves ophthalmopathy. Histologic examination of retrobulbar tissue samples shows proliferation of fibroblasts accompanied by accumulation of glycosaminoglycans. Connective tissue, fat, and muscles are infiltrated by mononuclear cells. (Weetman, Cohen et al. 1989; Heufelder, Bahn et al. 1993). These infiltrating cells are mainly T cells. (Weetman, Cohen et al. 1989; Heufelder, Bahn et al. 1993) Retrobulbar T cells from patients with Graves ophthalmopathy specifically recognize autologous retrobulbar fibroblasts. Retrobulbar fibroblasts from patients with Graves ophthalmopathy possess unique immunogenic properties, such as the constitutive expression of HLA class II molecules and of the heat shock protein hsp 72. (Heufelder, Gorman et al. 1991; Heufelder, Wenzel et al. 1991) Both molecules might support fibroblast recognition by T cells. (Grubeck-Loebenstein, Londei et al. 1988) These findings suggest that specific HLA antigens are involved in susceptibility to ophthalmopathy associated with GD.

CHAPTER IV

MATERIALS AND METHODS



Subjects

One hundred and twenty-four Thai patients with GD attending at King Chulalongkorn Memorial hospital were included in the study. One hundred and twenty-four normal controls for the population based case-control association study were recruited from volunteer unrelated healthy donors from the same geographic area. The study was approved by the ethics committee of the King Chulalongkorn University and the subjects gave their informed consent. Demographic data of the subjects was summarized in table .

DNA extraction

DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as anticoagulant, using a salting out method (Miller, Dykes et al. 1988). For the genomic DNA extraction, 1 ml of red cell lysis buffer (RCLB) was added to 0.5 ml of buffy coat, vortex for 30 seconds. This solution was centrifuged at 10,000-12,000 rpm for 30 seconds and the supernatant was discarded to obtain the pellet. The pellet remaining should be white to pink. This step may be repeated if necessary. To this pellet, 200 μl nuclei lysis buffer (NLB) and 50 μl 10% SDS were added. Pellet was broken up with pipet tip and vortex to get powdery, tiny flakes. The solution, 150 μl of NLB and 10 μl of proteinase K (10 mg/ml in H_2O stored frozen) were added, followed by incubation at 65°C for 2 hours. Precipitation of proteins was obtained by adding 175 μl of 5.3 M NaCl. This solution was centrifuged at 10,000-12,000 rpm for 15 minutes in microfuge. After centrifugation, the DNA in the supernatant was precipitated in 1 ml of cold absolute ethanol. Invert 6-10 times to precipitate DNA, it will appear as a white to translucent stringy mass. This solution was centrifuged at 10,000-12,000 rpm for 10 minutes and the supernatant was discarded to obtain the pellet. This pellet was resuspend in 1 ml of cold 70% ethanol (break pellet by tapping), followed by centrifugation 1-2 minutes at 10,000-12,000 rpm and the supernatant was discarded to obtain the pellet. After removal of the ethanol, the pellet was dried at 37°C with the cap open to evaporate the ethanol. This pellet was dissolved the in 200 μl of sterile distilled water, followed by incubation at 65°C for 15 minutes. Use gentle vortexing to resuspend. If clumps of undissolved DNA are present, it will be incubated at 65°C until completely resuspended.

HLA Typing

PCR-SSP analysis of HLA-DQA1

The genomic DNA of 124 patients with GD and 124 healthy controls were amplified with the use of the HLA-DQA1 gene specific primers described in Olerup et al (Olerup, Aldener et al. 1993) (Table6). Specific primers that amplified the 117- 196 bp within exon 2 of the HLA-DQA1 gene.

The reaction volume for the amplification reaction was 20 μl, containing 50 ng/μl genomic DNA, 0.1 μl of 5.0 U Taq polymerase (Promega or Gibco), 2 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 1.2 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM deoxynucletide triphosphates, 1 μl (20 pmol) of specific primers and 0.1 μl (20 pmol) of internal control primers. Internal control primers were used to check for successful PCR amplification. These primers amplify a third intron of HLA-DRB1 gene. 5'-primer C5 (19mer, TGC CAA GTG GAG CAC CCA A, Tm 60°C and 3'-primer C3 (20mer, GCA TCT TGC TCT GTG CAG AT, Tm 60°C gave rise to a 796-base pair (bp) fragment. Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The PCR protocol consisted of an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation (94°C, 20 seconds), annealing (65°C, 50 seconds) and extension (72°C, 20 seconds) and final extension at 72°C for 7 minutes. The resulting products were further analyzed by electrophoresis in 1.5 % Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 100 volts for 40 minutes and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD). Negative controls without DNA template were included in each experiment. The positive results of HLA-DQA1 gene and HLA-DRB1 gene showed band of 117-196 and 796 bp fragment, respectively. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Table 6 Nuclotide sequences, lengths, T_m and specificities of the PCR primer pairs used for identification of the DQA1 alleles by PCR-SSP technique (Olerup, Aldener et al. 1993)

5'name	sequence	mer	Tm	3' name	sequence	mer	Tm	size of PCR product	Amplified specificities of DQA1 allele
A'501	CAT gAA TTT gAT ggA gAT gAg g	22	62	A'301	ATg ATg TTC AAg TTg TgT TTT gC	23	62	149	0101,0104
A'502	ACg gTC CCT CTg gCC AgT A	19	62	A'301	ATg ATg TTC AAg TTg TgT TTT gC	23	62	172	0101,0102,0104
A'503	CAT gAA TTT gAT ggA gAT gAg C	22	62	A'301	ATg ATg TTC AAg TTg TgT TTT gC	23	62	149	0102,0103
A'504	Acg gTC CCT CTg gCC AgT T	19	62	A'301	ATg ATg TTC AAg TTg TgT TTT gC	23	62	172	0103
A'504	Acg gTC CCT CTg gCC AgT T	19	62	A'302	CAg gAT gTT CAA gTT Atg TTT TAg	24	64	170	0201
A'505	TTC ACT CgT CAg CTg ACC AT	20	60	A'303	CAA ATT gCg ggT CAA ATC TTC T	22	62	183	0301
A'506	TTC ACT CgT CAg CTg ACC AC	20	62	A'303	CAA ATT gCg ggT CAA ATC TTC T	22	62	183	0302
A'507	ACC CAT gAA TTT gAT ggA gAC	21	60	A'304	CAC ATA CCA TTg gTA gCA gCA	20	60	190	0401
A'502	ACg gTC CCT CTg gCC AgT A	19	62	A'305	AgT Tgg AgC gTT TAA TCA gAC	21	60	186	0501
A'504	Acg gTC CCT CTg gCC AgT T	19	62	A'306	ggT CAA ATC TAA ATT gTC TgA gA	23	62	117	0601

Table 7 Interpretation of the DQA1 typing result

primer HLA-DQA1	1	2	3	4	5	6	7	8	9	10
0101	+	+								
0102		+	+							
0103			+	+						
0104	+	+								
0201					+					
0301						+				
0302							+			
0401								+		
0501									+	
0601										+

The PCR products were analyzed to confirm the results of DQA1 genotyping by DNA sequencing. Generic primers for DQA1 are DQAAMP-A (5' ATG GTG TAA ACT TGT ACC AGT 3') and DQAAMP-B (5' TTG GTA GCA GCG GTA GAG TTG 3').

DNA sequencing

DNA sequencing were used to validate the results of DQA1 gene polymorphism by PCR-SSP methods. For direct cycle sequencing, 40 μl of the PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN Inc.) to obtain clean double-standed DNA amplificates. Cycle sequencing was performed on an ABI Prism 310 Genetic Analyzer using a cycle sequencing chemistry with base-specific fluorescence labeled dideoxynucleotide termination reagents, BigDye Terminator Ready Reaction Mix (Applied Biosystems) was used for sequencing. Thus, each sequencing reaction mixture of 10 μl final volume contained 1 μl of 5 pmol primer, 3 μl of template and 3 μl of the BigDye Terminator Ready Reaction Mix. Each sample mixture was then subjected to a cycle sequencing reaction in a Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The condition of cycle sequencing reaction consisting of denaturation at 96°C for 30 seconds, annealing at 55°C for 10 seconds and extension at 60°C for 4 minutes were carried out. Then each sequencing reaction product was pooled into 2 μl of 3 M sodium acetate (NaOAc, pH4.6) and 50 μl of 95% ethanol (EtOH) mixture in 1.5 microcentrifuge tubes, incubated at room temperature for 15 minutes to precipitate the extension products and centrifuged at 13,000 rpm for 20 minutes. The products were washed with 70% ethanol (EtOH) and centrifuged for 5 minutes at 13,000 rpm. The DNA pellet was then dried by place the tubes with the lids open in a heat block or thermal cycler at 90°C for 1 minute. Finally, the samples were resuspended in 15 μl of TSR (template suppression reagent), heat the samples at 95°C for 2 minutes and then chill on ice. The samples were loaded into an ABI Prism 310 Genetic Analyzer. Data collection was performed using the software package provided with the ABI 310 a sequencing system.

PCR-SSOP analysis of HLA-DQB1 and DRB1

With the exception of the DRA molecule, the genes encoding the fuctional Class II molecules are highly polymorphic with virtually all of the variability localised to the second exon. This exon, approximately 270 bp in length, encodes the amino-terminal extracellular domain which functions as the antigen binding site for processed peptides. Within this exon, the polymorphism is concentrated into discrete clusters which lie within a relatively conserved framework region. The DRB1 gene is the most polymorphic of the human Class II genes with the identification of over 400 distinct alleles.

Principles of the procedure

The Dynal RELI™ SSO HLA-DRB and HLA-DQB1 Test is based on three major processes: PCR target amplification, hybridisation of the amplified products to an array of immobilized sequence-specific oligonucleotide probes, and detection of the probe-bound amplified product by color formation.

PCR Amplification Reaction

For the HLA-DRB assay, a master mix containing a multiplex of five primers is used. One primer pair amplifies the specific target DNA contained in the polymorphic second exons of the DRB1, DRB3, DRN4 and DRB5 genes; each target sequence is 272bp in length. This primer pair also amplifies the DRB6 gene. However, the probes used in the assay are all specific for the polymorphic sequence motifs in the second exons of the DRB1, DRB3, DRB4 and DRB5 genes.

The master mix also contains an additional three primers which amplify DRB1*15 and DRB1*16 specific target sequences contained within intron 1 of the DRB1 gene; each target sequence is 114bp in length. DRB1*15 and DRB1*16 probes have been designed for this region and allow the assignment of this allele groups in the presence of DRB5 alleles.

For the HLA-DQB1 assay, the specfic target DNA sequence is the polymorphic second exon of the DQB1 gene; the target sequence is approximately 300 bp in length.

The DNA-containing specimen and reagent mixture are heated to 95°C to separate the double-stranded DNA and expose the target sequences to the primers. As the mixture cools, the biotinylated primers anneal to their targets. The Taq DNA polymerase in the presence of excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanoside, dedeoxycytidine and deoxyuridine, extends the annealed primers along the target templates to produce a biotinylated DNA sequence termed an amplicon. This process is repeated for a number of cycles, each cycle effectively doubling the amount of target DNA. For this test, the required number of cycles has been determined to be 35, theoretically yielding more than a billion-fold amplification.

Hybridization Reaction

After the PCR amplification process, the amplificons are chemically denatured to form single strands which are then added to a well of a typing tray that contains the nylon membrane with the immobilized, sequence-specific, oligonucleotide probes. The biotin-labeled amplicons then bind (hybridize) to the sequence specific probes and thus are captured onto the membrane strip. The stringent conditions for hybridization of the amplicons to the probes ensure the specificity of the reaction.

Detection Reaction

After a stringent wash of the membrane strip to remove unbound material, a streptavidin-horseradish peroxidase (SA-HRP) conjugate is added to the well of the tray. The streptavidin binds to the biotin-labeled amplicons captured by the membrane-bound probe. After washing off unbound conjugate, the bound SA-HRP conjugate is reacted with hydrogen peroxide (H_2O_2) and tetramethylbenzidine (TMB) to form a color

complex. The reaction is stopped by several water washes. The probe intensities are compared to the control probe and the typing pattern is recorded.

A positive Control DNA with a known HLA-DRB pattern (DRB1*0103 and DRB1*03011, 0305, 0306; DBR3*01011-01014, 0104) is supplied with the test kit.

A positive Control DNA with a known HLA-DQB1 type (DQB1*0201, *0202 and *0501) is supplied with the test kit.

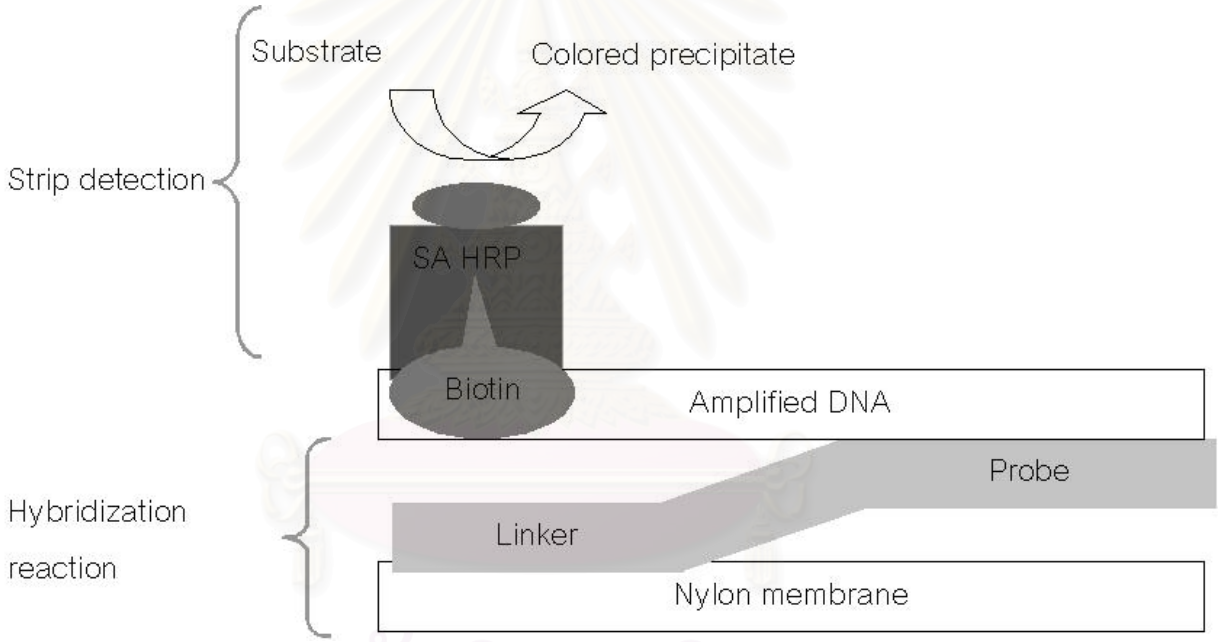


Figure 5 Principle of RELI™ SSO hybridization and detection assay

Statistical analysis

Allele and genotype frequencies

Allele and genotype frequencies were compared between groups using the Chi-square (χ^2) test or Fisher' s exact probability test, where appropriate. Gene frequencies were determined by gene counting. A *P* value of <0.05 was considered significant. Odds ratios (OR) with 95% confidence interval (CI) were calculated using the statistical program Epi Info version 6 [Centers for Disease Control and Prevention (CDC), 1994].

Haplotype analysis by PHASE program (Stephens and Donnelly 2003)

The program PHASE are used for reconstructing haplotypes from population genotype data. The remaining ambiguous sites are assigned by PHASE, and the uncertainty associated with each PHASE assignment is calculated (see Appendix IV).

Table 8 Characteristics of healthy controls and patients with GD

Characteristics	healthy controls	GD
Number of patients	124	124
Females/male	81/43	108/16
Mean age $\pm$ SD yr	23 $\pm$ 10.70	38 $\pm$ 12.41

CHAPTER V

RESULTS

PCR-SSP analysis of HLA-DQA1

Polymorphisms within second exon of the HLA-DQA1 gene were identified by the PCR-SSP method. The positive results of HLA-DQA1 gene and HLA-DRB1 gene (internal control) showed band of 117-196 and 796 bp fragment, respectively (Figure 6). In addition, direct sequencing were performed to validated the SSP results (data not shown).

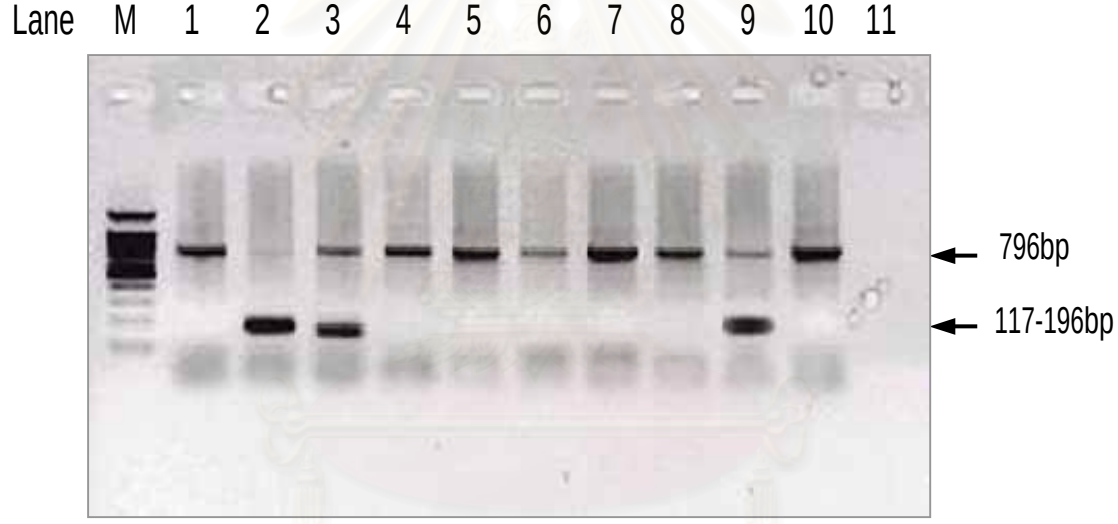


Figure 6 The representative result from 1 individaul with heterozygous of HLA-DQA1 gene

Lane M is 100 bp molecular marker.

Lane 1-10 (10 primers for one sample) all show positive internal control band.

Lane 2,3 and 9 show specific band that interpreted for HLA-DQA1*0102 (Lane 2 and 3) and DQA1*0501 (Lane 9). (based on Table 7)

Lane 11 is negative control (no DNA sample)

PCR-SSOP analysis of HLA-DRB and DQB1

HLA-DRB

One DRB primer pair amplifies the specific target DNA contained in the polymorphic second exons of the DRB1, DRB3, DRB4 and DRB5 genes; each target sequence is 272bp in length (Figure 7). The master mix also contains an additional three primers which amplify DRB1*15 and DRB1*16 specific target sequences contained within intron 1 of the DRB1 gene; each target sequence is 114bp in length (Figure 7). Then, The PCR products were hybridized with sequence specific oligonucleotide probes. For this Locus, there are 45 probes for detect DRB allele (Figure 8).

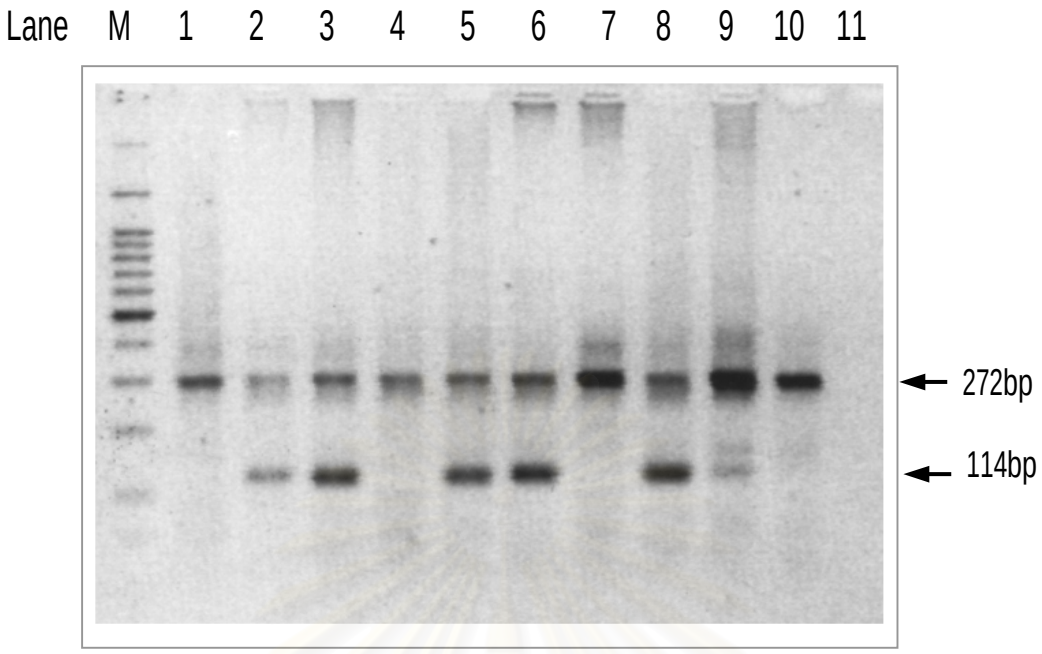


Figure 7 The representative result from 10 samples with HLA-DRB specific primers amplification

Lane M is 100 bp molecular marker.

Lane 1-10 samples all show DRB band (272bp)

Lane 2,3,5,6,8 and 9 show specific band (114bp) demonstrating that DNA sample contains HLA-DRB1*15 or DRB1*16

Lane 11 is negative control (no DNA sample)

The DRB type is assigned by reading the pattern of positive signals (blue lines) on the typing strip to determine which DRB alleles are present in the DNA samples (Figure 8). Results can be rercorded manually using the Overlay and Scoresheet provided with the typing kit. The interpretation Dynal RELI™ SSO pattern Matching Program provided is then used to assign a typing result (Figure 9).

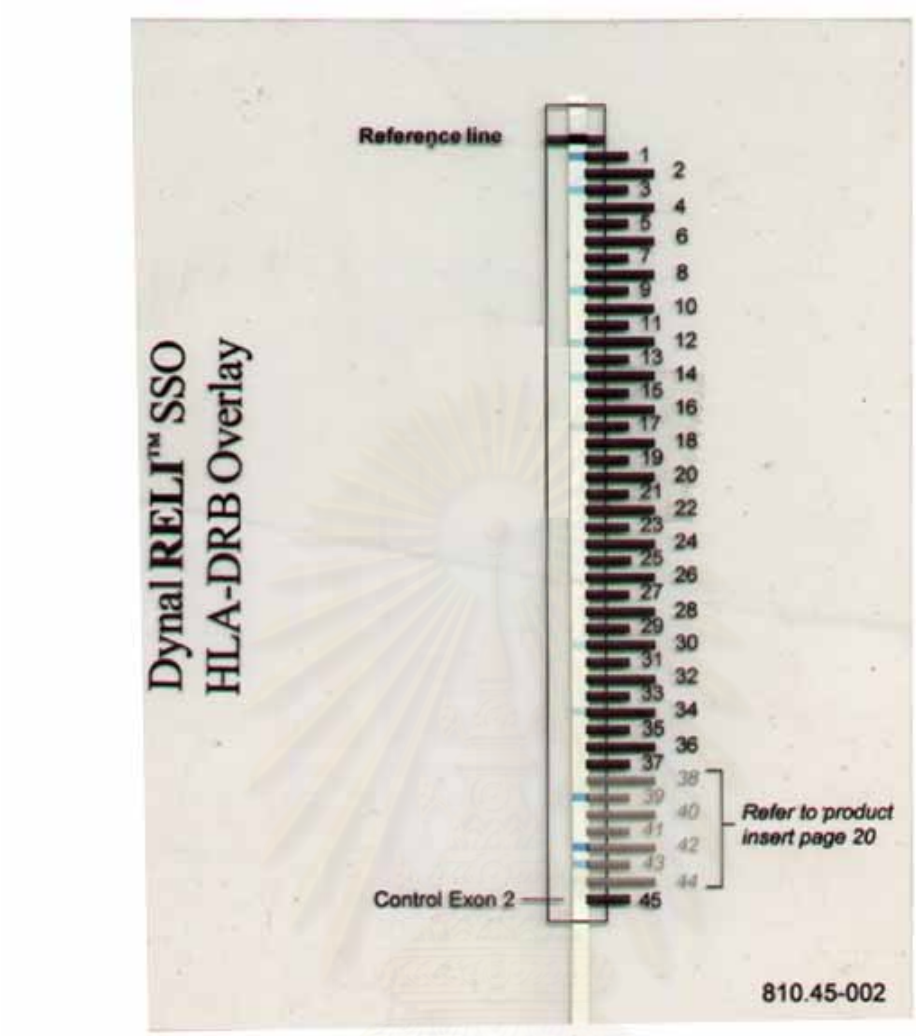


Figure 8 The representative result from 1 individual sample with HLA-DRB SSOP detection

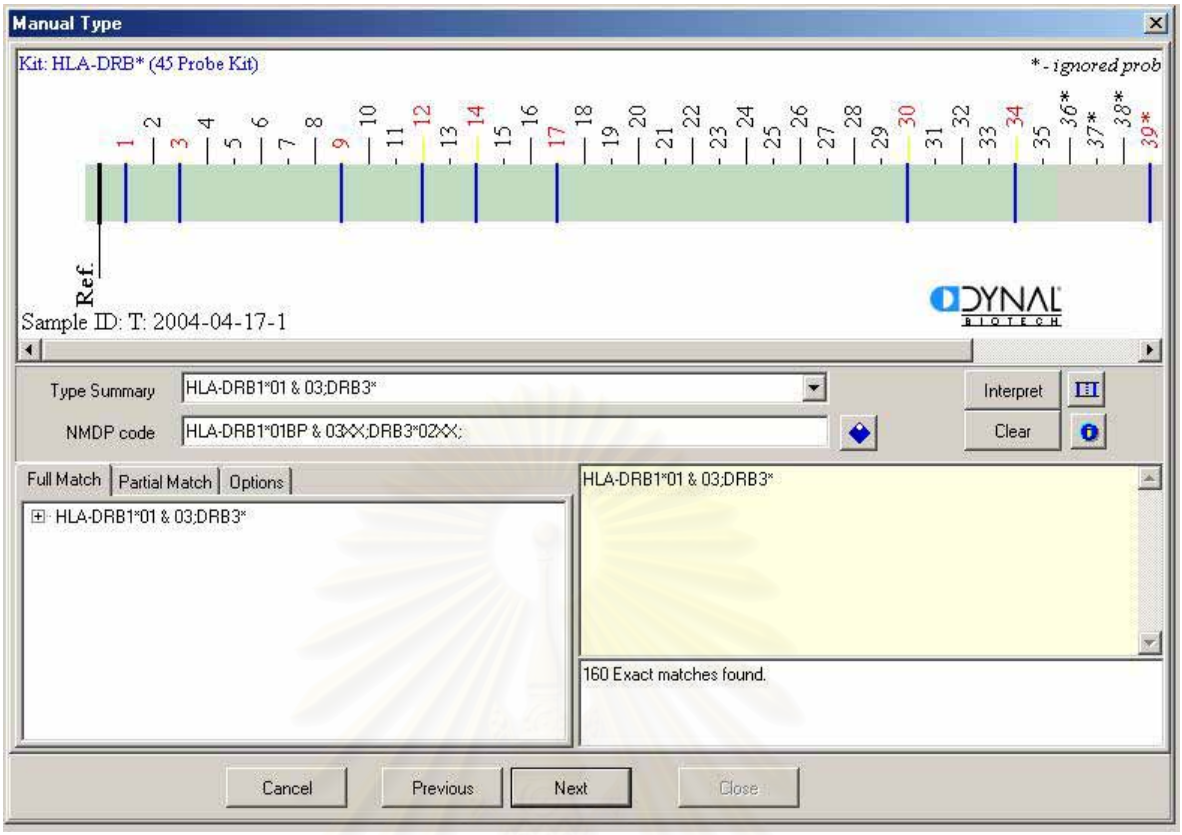


Figure 9 Dynal RELI™ SSO pattern Matching Program. The result was entered by selecting the positive probes in the strip display and choose interpret

HLA-DQB1

One DQB1-specific primer pair amplifies the specific target DNA contained in the polymorphic second exons of DQB1 genes; target sequence is 300bp in length (Figure 10). The PCR product were hybridized with sequence specific oligonucleotide probes. For this Locus, there are 25 probes to detect DQB1 allele (Figure 11).

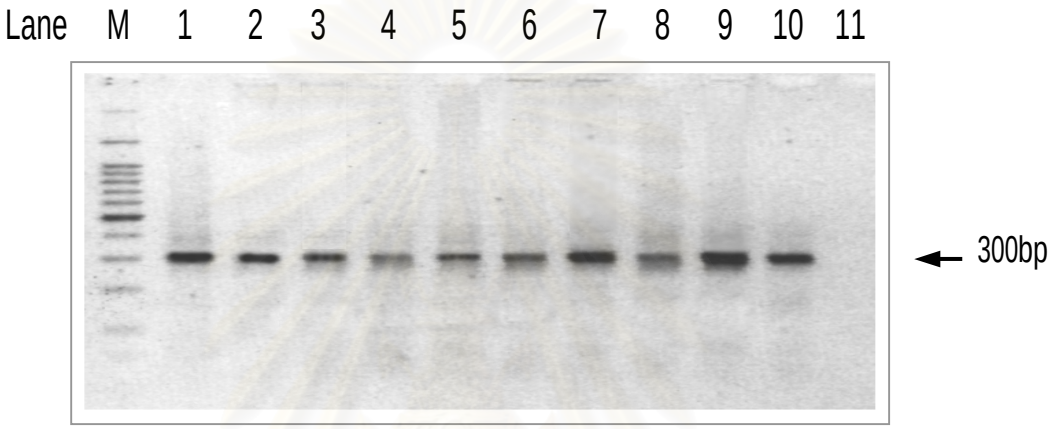


Figure 10 The representative result from 10 samples with HLA-DQB specific primers amplification

Lane M is 100 bp molecular marker.

Lane 1-10 samples all show DQB band (300bp)

Lane 11 is negative control (no DNA sample)

The same as DRB type, DQB1 type is assigned by reading the pattern of positive signals on the typing strip to determine which DQB1 alleles are present in the DNA samples (Figure 12). Results are interpreted by Dynal RELI™ SSO pattern Matching Program.

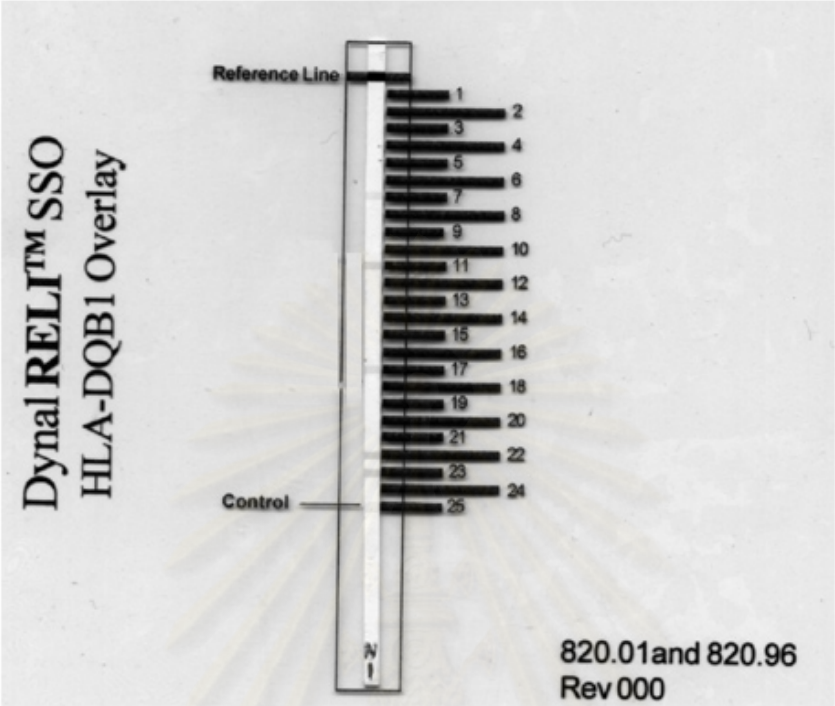


Figure 11 The representative result from 1 individual sample with HLA-DQB1 SSOP detectiion

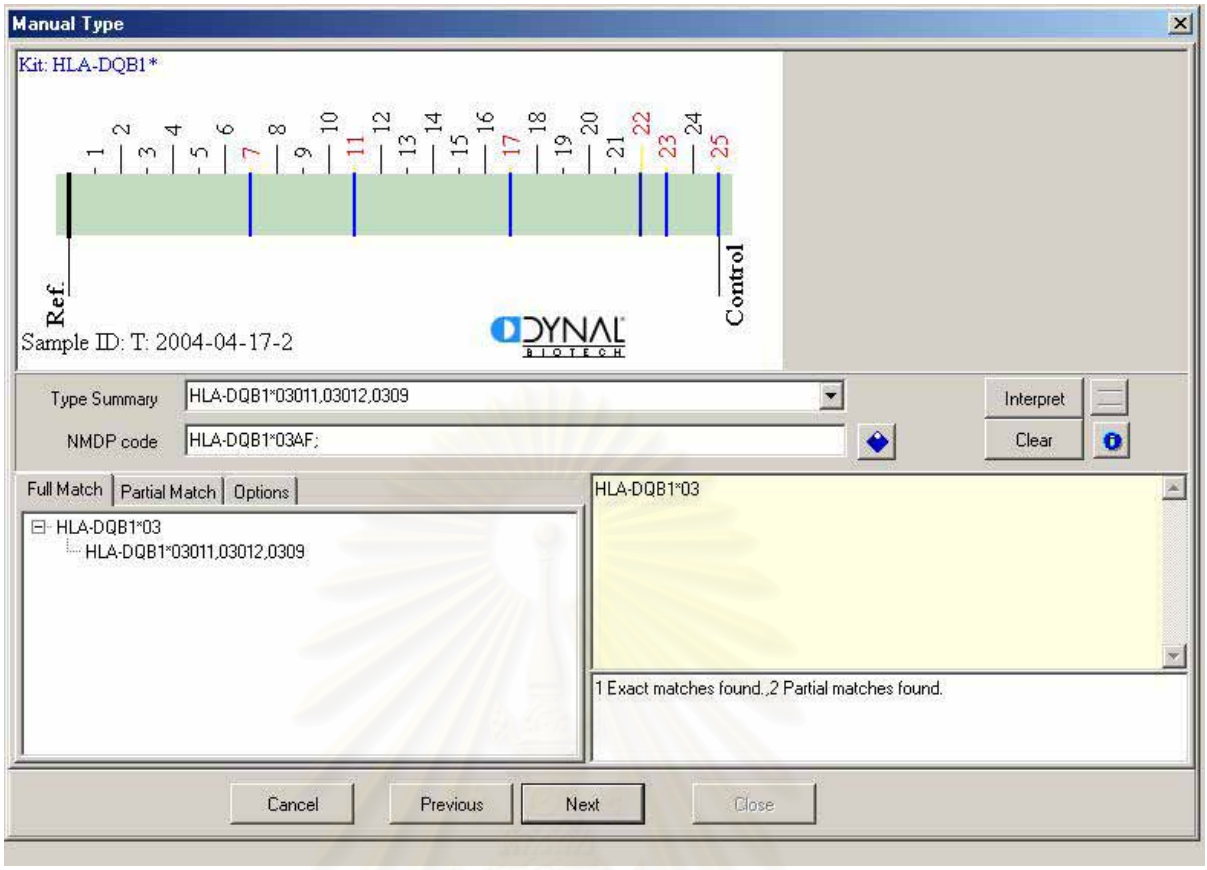


Figure 12 Dynal RELI™ SSO pattern Matching Program. The result was entered by selecting the positive probes in the strip display and choose interpret

Allele and Haplotype analysis

HLA-DQA1

The allele frequencies (AF) of DQA1 were shown in Table 9. Similarly, seven DQA1 alleles were identified in two groups. In control group, the common alleles (>10%) are DQA1*03 at an AF of 25%, DQA1*0102 at an AF of 0.19.4%, DQA1*0101/04 at an AF of 18.1% and DQA1*0601 at an AF of 10.5%. In patient group, the same four common alleles observed at high frequencies (14.1%-31.5%) excepted DQA1*0601 (4%).

The frequency of DQA1*0102 was significantly increased in patients with GD compared to the controls (31.5% vs. 19.4%; p = 0.002; OR = 1.91). Further analysis of DQA1 alleles showed a significant decrease in the frequency of the DQA1*0201 (2.0% vs. 7.7% in controls; p = 0.006, OR= 0.25) and DQA1*0601 alleles (4.0% vs. 10.5% in controls; p = 0.009, OR= 0.36) in the patients.

Table 9 Allele frequencies of HLA-DQA1 genes in GD patients and controls

HLA	Patients N:124 2n(%)	Controls N:124 2n(%)	*P*	OR	95%CI
DQA1*0101/04	42 (16.9)	45 (18.1)	NS		
*0102	78 (31.5)	48 (19.4)	0.002	1.91	1.24-2.95
*0103	13 (5.2)	9 (3.6)	NS		
*0201	5 (2.0)	19 (7.7)	0.006	0.25	0.08-0.72
*03	65 (26.2)	64 (25.8)	NS		
*0501	35 (14.1)	37 (14.9)	NS		
*0601	10 (4.0)	26 (10.5)	0.009	0.36	0.16-0.80

NS = not significant

HLA-DQB1

Fifteen DQB1 alleles were defined in control group. HLADQB1*0301 (at an AF of 20.2%), DQB1*0303 (at an AF of 14.1%), DQB1* 0201/0502 (at an AF of 13.3) and DQB1*0501 (at an AF of 10.1%) were commonly found in control group. DQB1*0603, *0605 and *0610 alleles were rare DRB1*0604 was not found in control samples but found only once in the patient group. Fourteen alleles were detected in GD patients with the same four common alleles observed at high frequencies (12.1%-23%) excepted DQA1*0201 (table 9). DQB1*0609 and *0610 alleles were not found in patient group.

The distribution of DQB1 allelic frequencies was significantly different between the GD and control groups. The frequency of DQB1*0502 was significantly increased in patients than controls (23.0% vs. 13.3%; p = 0.007; OR = 1.94). The frequency of DQB1*0201 and DQB1*0301 was slightly decreased in patients compared to the controls, but this difference was not significant.

Table 10 Allele frequencies of HLA-DQB1 genes in GD patients and controls

HLA	Patients N:124 2n(%)	Controls N:124 2n(%)	*P*	OR	95%CI
DQB1*0201	20 (8.1)	33 (13.3)	NS		
*0301	36 (14.5)	50 (20.2)	NS		
*0302	13 (5.2)	18 (7.3)	NS		
*0303	40 (16.1)	35 (14.1)	NS		
*0401	13 (5.2)	10 (4.0)	NS		
*0402	3 (1.2)	3 (1.2)	NS		
*0501	30 (12.1)	25 (10.1)	NS		
*0502	57 (23.0)	33 (13.3)	0.007	1.94	1.18-3.20
*0503	11 (4.4)	12 (4.8)	NS		
*0601	19 (7.7)	17 (6.9)	NS		
*0602	3 (1.2)	5 (2.0)	NS		
*0603	2 (0.8)	1 (0.4)	NS		
*0604	1 (0.4)	0 (0)	NS		
*0605	2 (0.8)	2 (0.8)	NS		
*0609	0 (0)	3 (1.2)	NS		
*0610	0 (0)	1 (0.4)	NS		

NS = not significant

HLA-DRB1

The allele frequencies of HLA-DRB1 and DQB1 are shown in Table 11. A total of 13 HLA-DRB1 alleles was observed in this study. The common alleles are DRB1*15 at an AF of 18.1%, DRB1*12 at an AF of 16.9%, DRB1*04 at an AF of 13.3% and DRB1*09 at an AF of 12.5%. DRB1*01 alleles was rare, being found in the three control samples and was absent in the GD samples. In patient group was detected the same three common alleles observed at high frequencies (12%-22.6%) excepted DRB1*1602. DRB1*10 alleles was rare in patient group.

Compared to the controls, the frequency of DRB1*1602 was significantly increased in patients (10.9% vs. 4.0%; p = 0.0063; OR = 2.91). In the DRB1 gene the frequency of allele DRB1*07 was significantly reduced in patients compared to the controls (2.0% vs. 7.7%; p = 0.006; OR = 0.25), providing a protective effect against GD.

Table 11 Allele frequency of HLA-DRB1 genes in GD patients and controls

HLA	Patients N:124 2n(%)	Controls N:124 2n(%)	P	OR	95%CI
DRB1*01	0 (0)	3 (1.2)	NS		
*03	17 (6.9)	17 (6.9)	NS		
*04	24 (9.7)	33 (13.3)	NS		
*07	5 (2.0)	19 (7.7)	0.0065	0.25	0.08-0.72
*08	7 (2.8)	5 (2.0)	NS		
*09	40 (16.1)	31 (12.5)	NS		
*10	1 (0.4)	6 (2.4)	NS		
*11	13 (5.2)	14 (5.6)	NS		
*12	29 (12.0)	42 (16.9)	NS		
*13	10 (4.0)	7 (2.8)	NS		
*14	19 (7.7)	16 (6.5)	NS		
*15	56 (22.6)	45 (18.1)	NS		
*16	27 (10.9)	10 (4.0)	0.0063	2.91	1.31-6.59

NS = not significant

Haplotye Analysis

Haplotype analysis was performed on the basis of the known association of DRB1, DQA1 and DQB1 alleles in Thais (Imanishi et al., 1992; Chandanayingyong et al., 1997; Stephens et al., 2000) and the results were confirmed by PHASE calculation software (Stephens and Donnelly 2003) (Table 12 and 13). Both methods reported similar results. Fifty-four haplotype patterns were detected in normal control group less than that in the patient group (67 haplotype patterns). The most common haplotypes were found with DR2 (DRB1*15 and *16) of both groups. Among DR2 haplotypes, DRB1*15-DQA1*0101-DQB1*0501 was the most common haplotype in the control groups (5.59%). Different from control group, DRB1*1602-DQA1*0102-DQB1*0502 was the most common haplotype in patient group (9.61%). There were 26 haplotypes of control group and 18 haplotypes of patient group showing frequencies of more than 1% as listed in Table 15 and Table 16. DRB1*09-DQA1*03-DQB1*0303 was found with highest frequency (12.5% in control and 15.7% in patient group).

Haplotype analysis revealed three haplotypes associated with GD (Table 14). DRB1*1602, DQA1*0102 and DQA1*0502 alleles provided susceptibility for GD with a OR of 2.91, 1.91 and 1.94 respectively. The DRB1*1602-DQA1*0102–DQB1*0502 haplotype was significantly increased in GD (9.6% vs. 4%, P = 0.0208, OR = 2.55) (Table 14). In contrast, The DRB1*07–DQA1*0201-DQB1*0201 and DRB1*12-DQA1*0601-DQB1*0301 haplotypes provided protection against GD (OR = 0.32 and 0.28 respectively).

Table 12 Haplotype frequency of normal controls

Index	Haplotype			Frequency	S.E.
	DRB1	DQA1	DQB1		
1	*01	*0101/04	*0501	0.012097	0
2	*03	*0501	*0201	0.064474	0.000409
3	*03	*0601	*0201	0.004074	0.000407
4	*04	*03	*0301	0.008386	0.001093
5	*04	*03	*0401	0.040001	0.001093
6	*04	*03	*0601	0.000081	0.000564
7	*04	*03	*0302	0.072580	0.000040
8	*04	*03	*0402	0.012016	0.000565
9	*07	*0201	*0201	0.060443	0.000402
10	*07	*0201	*0303	0.016129	0.000021
11	*08	*0103	*0601	0.020121	0.000401
12	*09	*03	*0303	0.124920	0.000564
13	*10	*0101/04	*0501	0.024153	0.000402
14	*10	*0102	*0502	0.000040	0.000401
15	*11	*0501	*0201	0.000970	0.001724
16	*11	*0501	*0301	0.055240	0.001877
17	*11	*0501	*0401	0.000241	0.000956
18	*12	*0501	*0301	0.020203	0.000407
19	*12	*0601	*0201	0.000040	0.000401
20	*12	*0601	*0301	0.104475	0.001156
21	*12	*0601	*0401	0.000080	0.000564
22	*12	*0102	*0301	0.004194	0.000791

Table 12 Haplotype frequency of normal controls (continued)

Index	Haplotype			Frequency	S.E.
	DRB1	DQA1	DQB1		
23	*12	*0102	*0502	0.032258	0.000026
24	*12	*0103	*0301	0.008104	0.001066
25	*13	*0501	*0201	0.003062	0.001724
26	*13	*0501	*0301	0.000970	0.001724
27	*13	*0601	*0603	0.000161	0.000790
28	*13	*0102	*0605	0.007943	0.000689
29	*13	*0102	*0609	0.012096	0.000033
30	*13	*0103	*0603	0.003871	0.000790
31	*13	*0103	*0605	0.000121	0.000689
32	*14	*0101/04	*0601	0.002469	0.002046
33	*14	*0101/04	*0502	0.019506	0.001488
34	*14	*0101/04	*0602	0.000364	0.001156
35	*14	*0101/04	*0503	0.040121	0.000962
36	*14	*0102	*0601	0.001604	0.001974
37	*14	*0102	*0502	0.000049	0.000443
38	*14	*0102	*0602	0.000242	0.000958
39	*14	*0102	*0503	0.000161	0.000881
40	*15	*0101/04	*0501	0.0559	0.0015
41	*15	*0101/04	*0601	0.005716	0.00214
42	*15	*0101/04	*0502	0.01637	0.000961
43	*15	*0101/04	*0602	0.004552	0.00137
44	*15	*0101/04	*0503	0.000081	0.000565
45	*15	*0102	*0501	0.008535	0.001295
46	*15	*0102	*0601	0.038517	0.002157

Table 12 Haplotype frequency of normal controls (continued)

Index	Haplotype			Frequency	S.E.
	DRB1	DQA1	DQB1		
47	*15	*0102	*0402	0.00008	0.000564
48	*15	*0102	*0502	0.0246	0.001508
49	*15	*0102	*0602	0.014963	0.001924
50	*15	*0102	*0503	0.003992	0.000697
51	*15	*0102	*0610	0.004032	0.000001
52	*15	*0103	*0503	0.004032	0
53	*16	*0101/04	*0501	0.000121	0.000689
54	*16	*0102	*0502	0.0402	0.000694

S.E. = standard deviations

Table 13 Haplotype frequency of GD patients

Index	Haplotype			Frequency	S.E.
	DRB1	DQA1	DQB1		
1	*03	*0101/04	*0501	0.000081	0.000564
2	*03	*0501	*0201	0.059879	0.001548
3	*03	*0501	*0301	0.008548	0.001537
4	*04	*03	*0301	0.000081	0.000565
5	*04	*03	*0401	0.052339	0.000564
6	*04	*03	*0501	0.00004	0.000401
7	*04	*03	*0302	0.040322	0.000033
8	*04	*03	*0402	0.003992	0.000403
9	*07	*0201	*0201	0.020161	0.000013
10	*08	*0103	*0301	0.004032	0
11	*08	*0103	*0601	0.024193	0.000025
12	*09	*03	*0302	0.004032	0.000005
13	*09	*03	*0303	0.157218	0.000401
14	*10	*0101/04	*0501	0.003952	0.000564
15	*10	*0501	*0201	0.000081	0.000564
16	*11	*0101/04	*0601	0.000323	0.001094
17	*11	*0101/04	*0503	0.002852	0.002003
18	*11	*03	*0602	0.00008	0.000564
19	*11	*03	*0303	0.003911	0.000688
20	*11	*0501	*0301	0.043025	0.00199
21	*11	*0102	*0301	0.001209	0.001848

Table 13 Haplotype frequency of GD patients (continued)

Index	Haplotype			Frequency	S.E.
	DRB1	DQA1	DQB1		
22	*11	*0102	*0601	0.000041	0.000405
23	*11	*0102	*0602	0.000041	0.000403
24	*11	*0102	*0503	0.000897	0.001677
25	*15	*0501	*0301	0.004032	0.000022
26	*15	*0501	*0402	0.004032	0
27	*12	*0601	*0301	0.035968	0.001391
28	*12	*0601	*0402	0.004032	0
29	*12	*0601	*0502	0.000242	0.001286
30	*12	*0102	*0301	0.020323	0.001418
31	*12	*0102	*0502	0.036129	0.001418
32	*12	*0102	*0602	0.002448	0.001969
33	*12	*0102	*0503	0.001584	0.001969
34	*12	*0103	*0301	0.004032	0
35	*13	*0501	*0201	0.00016	0.000788
36	*13	*0501	*0301	0.017137	0.001983
37	*13	*0501	*0402	0.000041	0.000403
38	*13	*0102	*0301	0.002823	0.001848
39	*13	*0102	*0604	0.004032	0.000006
40	*13	*0102	*0605	0.008064	0.000013
41	*13	*0103	*0603	0.008064	0.000018
42	*14	*0101/04	*0501	0.004072	0.000401
43	*14	*0101/04	*0601	0.005088	0.001773
44	*14	*0101/04	*0502	0.035395	0.002047

Table 13 Haplotype frequency of GD patients (continued)

Index	Haplotype			Frequency	S.E.
	DRB1	DQA1	DQB1		
45	*14	*0101/04	*0503	0.031976	0.001029
46	*14	*0501	*0301	0.00008	0.000563
47	*15	*0101/04	*0501	0.083415	0.002314
48	*15	*0101/04	*0601	0.000897	0.001677
49	*15	*0101/04	*0502	0.000041	0.000403
50	*15	*0101/04	*0503	0.000041	0.000405
51	*15	*03	*0303	0.000041	0.000403
52	*15	*0501	*0201	0.000323	0.001095
53	*15	*0501	*0301	0.003789	0.001252
54	*15	*0102	*0501	0.016705	0.001411
55	*15	*0102	*0601	0.037924	0.002862
56	*15	*0102	*0502	0.061944	0.002736
57	*15	*0102	*0602	0.009488	0.002206
58	*15	*0102	*0303	0.000121	0.000687
59	*15	*0102	*0503	0.002972	0.002849
60	*15	*0103	*0501	0.004032	0.000002
61	*15	*0103	*0601	0.008064	0.000013
62	*16	*0101/04	*0501	0.001181	0.002286
63	*16	*0102	*0301	0.000080	0.000563
64	*16	*0102	*0501	0.007489	0.001411
65	*16	*0102	*0601	0.000081	0.000567
66	*16	*0102	*0502	0.096007	0.001894
67	*16	*0102	*0503	0.004032	0.000018

Table 14 Significant association of HLA-DRB1, DQA1, and DQB1 haplotype in GD patients and controls

Haplotype	Patients N:124 2n(%)	Controls N:124 2n(%)	*P*	OR	95%CI
DRB1-DQA1-DQB1					
16-0102-0502	24 (9.6)	10 (4.0)	0.0208	2.55	1.13-5.85
07-0201-0201	5 (2.0)	15 (6.0)	0.0399	0.32	0.10-0.96
12-0601-0301	8 (3.5)	26 (10.4)	0.0025	0.28	0.12-0.68

Table 15 The most common haplotype frequencies of control group (high to low frequency)

Index	Haplotype			Frequency
	DRB1	DQA1	DQB1	
1	*09	*03	*0303	0.125
2	*12	*0601	*0301	0.104
3	*04	*03	*0302	0.073
4	*03	*0501	*0201	0.064
5	*07	*0201	*0201	0.060
6	*15	*0101/04	*0501	0.056
7	*11	*0501	*0301	0.055
8	*16	*0102	*0502	0.040
9	*14	*0101/04	*0503	0.040
10	*04	*03	*0401	0.040
11	*15	*0102	*0601	0.039
12	*12	*0102	*0502	0.032
13	*15	*0102	*0502	0.025
14	*10	*0101/04	*0501	0.024
15	*12	*0501	*0301	0.020
16	*08	*0103	*0601	0.020
17	*10	*0101/04	*0501	0.024
18	*12	*0501	*0301	0.020
19	*08	*0103	*0601	0.020
20	*14	*0101/04	*0502	0.020
21	*15	*0101/04	*0502	0.016
22	*07	*0201	*0303	0.016
23	*15	*0102	*0602	0.015
24	*01	*0101/04	*0501	0.012
25	*13	*0102	*0609	0.012
26	*04	*03	*0402	0.012

Table 16 The most common haplotype frequencies of patient group (high to low frequency)

Index	Haplotype			Frequency
	DRB1	DQA1	DQB1	
1	*09	*03	*0303	0.157
2	*16	*0102	*0502	0.096
3	*15	*0101/04	*0501	0.083
4	*15	*0102	*0502	0.062
5	*03	*0501	*0201	0.060
6	*04	*03	*0401	0.052
7	*11	*0501	*0301	0.043
8	*04	*03	*0302	0.040
9	*15	*0102	*0601	0.038
10	*12	*0102	*0502	0.036
11	*12	*0601	*0301	0.036
12	*14	*0101/04	*0502	0.035
13	*14	*0101/04	*0503	0.032
14	*08	*0103	*0601	0.024
15	*12	*0102	*0301	0.020
16	*07	*0201	*0201	0.020
17	*13	*0501	*0301	0.017
18	*15	*0102	*0501	0.017

Distribution 0f HLA-DQA1, DQB1 and DRB1 alleles in various populations.

Besides association study of HLA gene polymorphisms with GD, this study also provides the basic knowledge of the frequency of HLA gene polymorphisms in Thai population. The distributions of the HLA gene polymorphisms between Thai population and other populations previously report were compared.

HLA-DQA1 alleles in five different populations

When comparing the distribution of HLA-DQA1 alleles between study population and Southern China population, no significant difference could be found (Table 17). The analysis showed significant difference between the study populations and Iran, Caucasian and African American populations ($\chi^2 = 39.77$, $p < 0.0001$; $\chi^2 = 47.68$, $p < 0.0001$; $\chi^2 = 28.20$, $p = 0.0004$), respectively (Table 17). DQA1*0401 allele was found in all populations but absent in Thai populations.

Table 17 HLA-DQA1 allele frequencies distribution in various populations

HLA-DQA1 allele	Thai (present study) N = 124 (%)	Southern Chinese N = 270 (%)	Iran[a] N = 36 (%)	Caucasian[b] N = 246 (%)	African American[c] N = 132 (%)
*0101/04	18.1	9.6	13.9	12.5	25.8
*0102	19.4	23	12.5	26.3	15.2
*0103	3.6	5.9	6.9	6.6	3
*0201	7.7	4.8	15.3	7.2	10.6
*03	25.8	28.5	8.4	3	15.9
*0401	0	0.7	4.2	0.2	7.6
*0501	14.9	15.2	38.9	39.1	18.2
*0601	10.5	12.2	0	0.2	0
others	0	0	0	4.6	3.8

[a] $\chi^2 = 39.77$, $p < 0.0001$; compare between allele frequencies in Thai with Iran (Dehaghani, Amirzargar et al. 2002)

[b] $\chi^2 = 47.68$, $p < 0.0001$; compare between allele frequencies in Thai with Caucasian (Papassavas, Spyropoulou-Vlachou et al. 2000)

[c] $\chi^2 = 28.20$, $p = 0.0004$; compare between allele frequencies in Thai with African American (Gregoire, Reidy et al. 2003)

Not significant ; compare between allele frequencies in Thai with Southern Chinese (Sun, Gao et al. 1991)

HLA-DQB1 alleles in five different populations

The distribution of DQB1 alleles between the five different populations is shown in Table 18. The results from this study and previous study in Thai population were similar in the distribution and the common alleles such as frequency of DQB1*0301/04, 0303, 0201 and 0502 (Sirikong, Tsuchiya et al. 2002). No significant differences were seen in the DQB1 frequency among the two studies in Thai populations. When compared the distribution of study population with Southern Chinese and Iran populations, no significant difference could be found. However, the analysis showed significant difference between the study population and Caucasian and African American populations ($\chi^2 = 23.09$, $p = 0.0269$; $\chi^2 = 66.847$, $p < 0.0001$).

Table 18 HLA-DQB1 allele frequencies distribution in various populations

HLA-DQB1 allele	Thai (present study) N = 124 (%)	Thai (previous study) N = 187 (%)	Southern Chinese N = 270 (%)	Iran N = 36 (%)	Caucasian[a] N = 246 (%)	African American[b] N = 132 (%)
*0201	13.3	12.8	10	23.6	12.8	21.2
*0301/04	20.2	23.5	21.9	38.9	32.5	12.1
*0302	7.3	6.7	7		7.1	0.8
*0303	14.1	13.1	16.3		2.6	4.5
*030X	0	0	0	0	0.4	7.6
*0401	4	3.7	5.6	5.6	0.2	0
*0402	1.2	0	0.7		0.4	10.6
*0501/04	10.1	9.9	3.7	19.4	8.5	11.4
*0502	13.3	13.6	10.4		16.3	0
*0503	4.8	3.5	3.3		4.5	3
*050X	0	0	0	0	0	3.8
*0601	6.9	9.4	11.1	4.2	2.6	1.5
*0602	2	1.3	5.9	6.9	3.7	14.4
*0603	0.4	1.1	2.2		5.1	0.8
*060X	2.4	0	1.9	1.4	3.2	8.4
others	0	1.3	0	0	0	0

[a] $\chi^2 = 23.09$, $p = 0.0269$; compare between allele frequencies in Thai with Caucasian (Papassavas, Spyropoulou-Vlachou et al. 2000)

[b] $\chi^2 = 66.85$, $p < 0.0001$; compare between allele frequencies in Thai with African American (Gregoire, Reidy et al. 2003)

Not significant ; compare between allele frequencies in Thai with Southern Chinese (Sun, Gao et al. 1991), Iran (Dehaghani, Amirzargar et al. 2002) and previous study in Thai (Sirikong, Tsuchiya et al. 2002).

HLA-DRB1 alleles in five different populations

The analysis of DRB1 gene showed no significant differences in allele frequencies between study population with previous study in Thai and Southern China populations (Sirikong, Tsuchiya et al. 2002). The most common alleles in Thai populations are DRB1*15, 12 and 09 that similar to Southern Chinese population. There were significant differences in allele frequencies between Thai population with Iran, Caucasian and African American populations ($\chi^2 = 43.54$, $p < 0.0001$; $\chi^2 = 65.55$, $p < 0.0001$; $\chi^2 = 47.40$, $p < 0.0001$, respectively).

Table 19 HLA-DRB1 allele frequencies distribution in various populations

HLA-DRB1 allele	Thai (present study) N = 124 (%)	Thai (previous study) N = 187 (%)	Southern Chinese N = 270 (%)	Iran[a] N = 36 (%)	Caucasian[b] N = 246 (%)	African American[c] N = 41 (%)
*01	1.2	0.0	0.4	2.8	7.5	5.3
*03	6.9	4.5	6.3	6.9	6.5	14.2
*04	13.3	6.0	11	9.7	9.1	3.8
*07	7.7	11.2	4.8	11.1	2.6	9.8
*08	2.0	2.7	4	4.2	8.5	8
*09	12.5	10.2	15.6	4.2	0.6	2
*10	2.4	1.6	0.7	2.8	1.2	1.9
*11	5.6	5.1	4.5	29.2	29.6	13.3
*12	16.9	18.7	17.8	1.4	1	5
*13	2.8	1.1	2.3	5.6	8.9	15.7
*14	6.5	5.4	4.8	8.3	4.5	1.5
*15	18.1	19.0	18.1	6.9	8.8	13.2
*16	4.0	4.3	6.3	6.9	13.5	2.7
others	0.0	6.7	3.4	0	0	3.6

[a] χ^2 =5.4, p=0.02 ; compare between allele frequencies in Thai with Iran (Dehaghani, Amirzargar et al. 2002)

[b] χ^2 =5.8, p=0.01 ; compare between allele frequencies in Thai with Caucasian (Papassavas, Spyropoulou-Vlachou et al. 2000)

[c] χ^2 =5.8, p=0.01 ; compare between allele frequencies in Thai with African American (Kuffner, Whitworth et al. 2003)

Not significant ; compare between allele frequencies in Thai (Sirikong, Tsuchiya et al. 2002) with Southern Chinese (Sun, Gao et al. 1991)

CHAPTER VI

DISCUSSION

Linkage studies of HLA in AITD have been largely negative (Bode, Dorf et al. 1973; Hawkins, Ma et al. 1985; Roman, Greenberg et al. 1992; Barbesino, Tomer et al. 1998). Only one recent study from the United Kingdom showed weak evidence for linkage between GD and the HLA region (Vaidya, Imrie et al. 1999), and an additional study reported linkage only when conditioning on DR3 (Shields, Ratanachaiyavong et al. 1994). Linkage analysis approach is a powerful tool for detecting major genes in complex diseases. However, linkage studies are less sensitive than association studies because they do not detect less influential genes and it is difficult to collect samples from families that include both parents.

Population-base case-control study, association study, is more sensitive than linkage study and therefore are better for fine-mapping linked genetic regions. Association analysis is highly sensitive and may detect genes contributing less than 5% of the total genetic contribution to a disease (Risch and Merikangas 1996). The problem of this approach is false positive association. This population-based association method may produce false positive associations if the patients and controls are not accurately matched. In this study, the HLA class II allele distribution of control group was compared with known allele frequency in Thai population (Sirikong, Tsuchiya et al. 2002) and found similar HLA allele distribution help exclude the genotyping error in our study.

In Caucasians HLA-DR3 was found to be consistenly associated with GD (Table 3). A recent family-based study from the United Kingdom using TDT analysis confirmed the results of the case control studies (Heward, Allahabadia et al. 1998). In non-Caucasians the HLA genes were shown to be associated with GD, as well, although the associated alleles varies among various ethnic background (Table 3).

In Thai population, DR3 allele could not be detected as susceptibility gene but unique alleles and haplotypes of DRB1*1602-DQA1*0102-DQB1*0502 were shown to be associated with GD as discussed in detail below. Tsai et al. demonstrated by serological testing that HLA-DR2 (contains DRB1*15 and DRB1*16 allele) had an additive role in the susceptibility of Taiwanese Chinese to GD (N = 93). The study in Taiwanese Chinese was repeated again by Huang and co workers. They collected samples with higher number (N = 236) and use DNA-based HLA typing method for higher resolution study. However their results could not identify the allele of DR2 susceptibility gene for GD. The HLA polymorphism in the DNA sequence level is a better indicator to study the association between HLA and autoimmune disease. It is known that different HLA alleles have different affinities for peptides from autoantigens (e.g., thyroid antigens) that are recognized by T cell receptors on cells that have escaped tolerance (Nelson and Hansen 1990). Therefore, DNA-based typing of HLA by PCR-SSP and PCR-SSOP technique were applied in this study.

The results showed highest of OR (2.91) in DRB1*1602 allele when compared with DQA1*0102 (OR = 1.91) and DQB1*0502 (OR = 1.94). When analyzed the haplotype frequency, we found the OR of DRB1*1602-DQA1*0102-DQB1*0502 haplotye (OR = 2.55) was less than OR analyzed from DRB*1602 (OR = 2.91) allele frequency. This observed suggests the significant association between allele with GD more than haplotype. The association with DQA1 and DQB1 migth be the result from linkage disequilibrium of DRB1. For example, while DRB1*1602-DQA1*0102-DQB1*0502 was significantly increased in GD (9.6% vs 4%; p = 0.02), but DRB1*12-DQA1*0102-DQB1*0502 and DRB1*15-DQA1*0102-DQB1*0502 were not significantly increased (3% vs 3%; p = NS and 2.5% vs 6%; p = NS). Taken together, this haplotype analyzes suggested that DRB1 alleles are primarily associated with susceptibility to GD, rather than DQ alleles. Although the haplotype analysis and OR value suggested a primary contribution of DRB1*1602 rather than DQ, the possibility that other gene(s) which are in linkage disequilibrium with DRB1 possess the primary role cannot be excluded.

There are at least two possible hypotheses for the existence of an association between an allele and a disease: 1) the associated allele itself is the genetic variant causing an increased risk for the disease; and 2) the associated allele itself is not causing the disease but rather a gene in linkage disequilibrium with it (Hodge 1994). The studies support the first hypothesis were performed by Sawai and DeGroot. They demonstrated that DR3 molecules bind TSHR immuonodominant peptide epitopes with intermediate affinity. They suggest that susceptibility to GD associated with inheritance of a specific HLA class II gene is due to the influence of the HLA molecule-TSHR peptide complex on the T cell repertoire. Ban et al. recently subtyped, by direct sequencing, HLA-DR3β chain in a population of DR3-positive GD patients and controls to identify critical amino acids for the susceptibility to GD (Ban, Davies et al. 2004). The allelic frequency of DRB1*0311 was significantly lower in patients than in controls. The specific amino acids occupying the peptide binding pockets of DRB1*0311 showed that the lack of arginine at position 74 of the DRβ chain (DRβ 74^{Arg}) was significantly more frequent in the DR3-positive controls than in the DR3-positive patients (Ban, Davies et al. 2004). These results suggested that GD is associated with specific sequences of the DR3 allele (possible mechanism show in Figure 14). However, DR3 is not the susceptibility allele in Thai population. DR3 subtype migth be required to determine the frequency of protective DRB1*0311 in Thai population. However, when we compare DRB1*03 and DRB1*1602 sequences which is the susceptibility allele in Thai GD, the arginine at position 74 is not found in DRB1*1602. The difference of amino acid at other position in DRB1*1602 may contribute to susceptibility to GD. Further studies such as the characteristics of TSHR peptides binding to these HLA class II molecules should be performed (Figure 13).

Interestingly, the lack of arginine at position 74 of DRB*0311 similar to DRB1*07 that is the protective allele in Thai population and several other studies (Lavard, Madsen et al. 1997) (Schmidt, Verdaguer et al. 1997). The arginine 74 is replaced with a glutamine, which is not only polar, but also has a shorter sidechain than does arginine. Glutamine is substituted in this position and may has a role in protective of GD. Besides DRB1*07, two protective haplotypes of DRB1*07-DQA1*0201-DQB1*0201 and

DRB1*12-DQA1*0601-DQB1*0301 were reported in this study. It is possible that DQA1 and DQB1 alleles as well as other genes in linkage disequilibrium to this haplotype might contribute to the protective effect in GD.

The second hypothesis should not be disregard. Some genes that link with HLA class II may contribute to disease development. Case-control studies have also shown an association of GD with alleles of several different genes within the MHC, including the HSP70, TNF, TAP and LMP genes (Ratanachaiyavong, Demaine et al. 1991; Badenhoop, Schwarz et al. 1992; Rau, Nicolay et al. 1997; Heward, Allahabadia et al. 1999; Hunt, Marshall et al. 2001). Due to the strong linkage disequilibrium of genes within the region, it is difficult to determine the independent effect of particular allele in disease susceptibility.

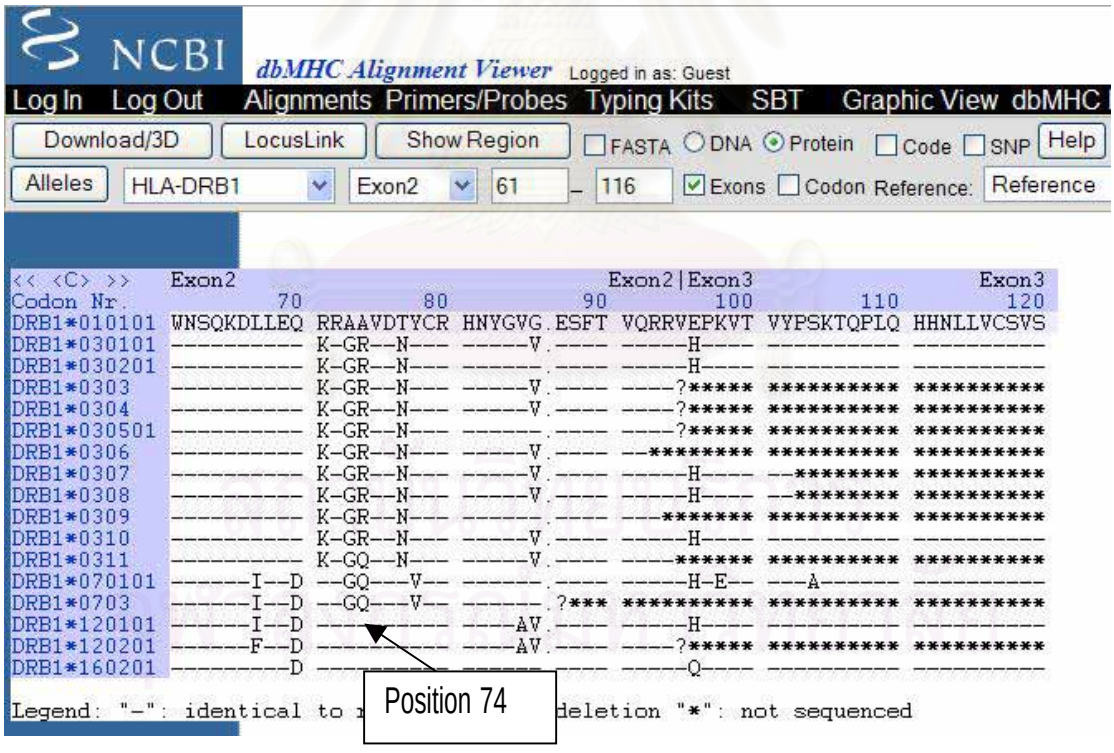


Figure 13 Alignment viewer for DRB*0301-0311, *1602, *0701 and *1201-02 (http://www.ncbi.nlm.nih.gov/mhc/)

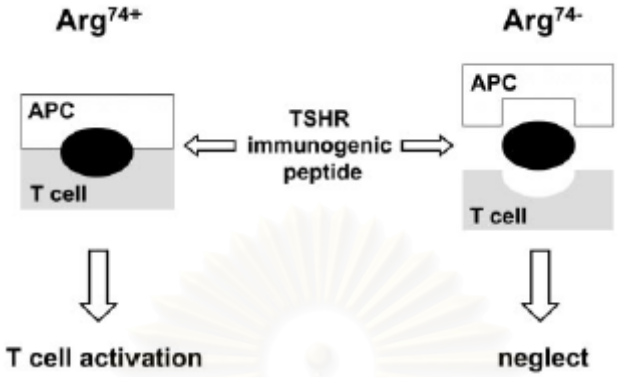


Figure 14 Ban et al. propose the possible mechanism of induction of GD by specific HLA-DR sequences. HLA-DR molecules containing Arginine at position 74 of the DRβ1 chain (DRβ 74Arg) form a peptide binding pocket that enables presentation of TSHR immunogenic peptides which stimulate TSHR-specific T cells. In contrast, HLA-DRβ molecules lacking the Arginine at position 74 of the DRβ 1 chain cannot fit the TSHR immunogenic peptides, and therefore TSHR-specific T cells are not stimulated (Ban, Davies et al. 2004).

In addition, our study showed the basic knowledge of the diversity of HLA class II in Thailand. Class II distribution in this study was similar to previously report in Southern Chinese (Sun, Gao et al. 1991) but different with Iran (Dehaghani, Amirzargar et al. 2002), Caucasian (Papassavas, Spyropoulou-Vlachou et al. 2000) and African-American (Gregoire, Reidy et al. 2003; Kuffner, Whitworth et al. 2003). In the present study, the data of class II allele frequencies reveal the limited polymorphism of class II with some difference patterns in the distribution of alleles in ethnically r populations.

CHAPTER VII

CONCLUSION

The aim of this research was to explore risk association between HLA class II gene polymorphisms and GD susceptibility. This study emphasizes the value of genetic variation in the human population in eliciting new risk factors and/or confirming earlier observations. The results demonstrated that the HLA-DRB1*1602-DQA1*0102-DQB1*0502 alleles and haplotype are the marker for genetic susceptibility to GD in Thai population. The risk association was detected for HLA-DRB1*1602-DQA1*0102-DQB1*0502 haplotype with a significant OR of 2.55 (95% CI = 1.13-5.85) and p = 0.02. In addition we found the protective association between DRB1*07-DQA1*0201-DQB1*0201 and DRB1*12-DQA1*0601-DQB1*0301 haplotypes with GD in Thai population (OR = 0.32, 95% CI = 0.10-0.96, p = 0.039 and OR = 0.28, 95% CI = 0.12-0.68, p = 0.002, respectively). Two major possibilities about the role of HLA class II in GD development can be hypothesized. One possibility is that the HLA-DRB1*1602, DQA1*0102 or DQB1*0502 alleles present self-antigen and activate autoreactive T cells better than other HLA class II alleles. This presentation of antigen might exacerbate the autoimmune processes involved in the pathogenesis of GD. However, further studies on the functionality of these alleles are necessary. Another possibility is the that HLA-DRB1*1602-DQA1*0102-DQB1*0502 haplotype might be closely linked with the susceptibility gene that contributes to GD development. Further study of other candidate genes within this region are also necessary.

Moreover, this study helps reveal HLA-DRB1, DQA1 and DQB1 polymorphism in Thailand. Thai population contain allelic polymorphism similar to ethnic groups from Chinese population. However, different pattern of alleles distribution was found when compared to Iran, Caucasian and African population.

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APPENDICES

APPENDIX I

Reagent for agarose gel electrophoresis

1. 50x Tris-acetate buffer (TAE)

Tris base	424.0	g
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	100	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

2. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the solution and store in the dark at 4°C.

3. 1.5% Agarose gel

Agarose	0.3	g
1x TAE	20	ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

4. 5x Loging buffer 100 ml

Tris HCL	0.6	g
EDTA	1.68	g
SDS	0.5	g
Bromphenol Blue	0.1	g
Sucrose	40	g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5 microtube and store at 4°C.

APPENDIX II

Reagent for DNA extraction

1. Red Cell Lysis Buffer (RCLB)

NH_4Cl	1.875	g
Tris-HCl	0.25	g

Dissolve NH_4Cl and Tris-HCL in 500 ml of distilled water. Adjust pH to 7.2. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated. Shelf life is approximately 6 months.

2. Nuclei Lysis Buffer (NLB)

1 M Tris (pH 8.0)	10	ml
5 M NaCl	0.5	ml
0.5 M EDTA (pH 8)	0.4	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.

3. 1 M Tris

Tris base	12.11	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

4. 5 M NaCl

NaCl	29.22	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

5. EDTA

EDTA	37.22	g
Distilled water	200	ml

Adjust volume to 200 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated.

6. 5.3 M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

7. Proteinase K 10 mg/ml

Proteinase K	100	mg
Distilled water	10	ml

Mix the solution and store at -20°C.

8. 10% SDS

SDS	10	g

Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

APPENDIX III

Reagent for PCR-SSOP hybridization
Dynal RELI™ SSO HLA-DRB Typing Kit Product Code 810.45

Table 24 Kit contents

Dynal RELI™ SSO HLA-DRB Typing Kit Product Code 810.45	Description
1. HLA-DRB Master Mix	A Tris HCl solution containing: 10% glycerol, 100mM KCl, <0.001% AmpliTaq® (Taq polymerase) 0.05% sodilum azide (as a preservative).
2. Control DNA 6 tests	DNA from a B lymphoboasoid cell in a Tris-HCl, EDTA solution containing 0.05% sodium azide.
3. HLA-DRB Typing Strips	50 strips in a resealable glass tube sith dessiccant.
4. 6.0mM $MgCl_2$Strips	A 6.0mM magnesium chloride solution containing 1% ProClin® 300 (as a preservative) ProClin® 300 is an irritant.
5. HLA –DRB Scoresheets	A pad for scoring positive probes.
6.HLA-DRB Overlay	A clear plastic overlay for determining probe Positions.

Table 25 Strip detection kits

Dynal RELI™ SSO Strip Detection Kits	Description
1. Denaturaturation Solution	A solution containing 3% EDTA, 1.6% sodium Hydroxide and thymol blue 1.6% sodium hydroxide is an irritant.
2. SSPE Concentrate	Sodium phosphate solution with NaCl, EDTA and 1.0% ProClin® 150 (as a preservative) ProClin® 150 is an irritant.
3. SDS Concentrate	Sodium dodecyo sulphate soltion with 1.0% ProClin® 150.
4. Streptavidin- HRP Conjkugate	Streptavidin-horseradish peroxidase Conjugate in an ACES solution with NaCl and 1.0% ProClin® 150
5. Substrate A	A citrate solution containing 0.01% H_2O_2 and 0.01% ProClin® 150.
6. Substrate B	Contains 0.1% 3,3',5,5'-tetramethylbenzidine (TMB) in 40% dimethylformamide (DMF) DMF is Toxic.
7. Citrate Concentrate	Sodium citrate solution.

APPENDIX IV

PHASE program is produced by the Mathematical Genetics Group, University of Oxford, Oxford, UK. The software is available online at http://www.stats.ox.ac.uk/ mathgen/ (Stephens and Donnelly 2003).

Input file format

The input file is supplied by the user to specify how many individuals there are to be analysed, how many loci/sites each individual has been typed at, what sort of loci/ sites these are (SNP or microsatellite), and the genotypes for each individual. The default format input file, as illustrated in the accompanying file test.inp. The default structure for the input file can be represented as follows:

NumberOfIndividuals
NumberOfLoci
P Position(1) Position(2) Position(NumberOfLoci)
LocusType(1) LocusType(2) ... LocusType(NumberOfLoci)
ID(1)
Genotype(1)
ID(2)
Genotype(2)

.

ID(NumberOfIndividuals)]
Genotype(NumberOfIndividuals)

Where the quantities above are as follows:

- Number Of Individuals An integer specifying the number of individuals who have been genotyped.

- Number Of Loci An integer specifying the number of loci or sites at which each individual has been typed.

- P The character 'P' (upper case, without quotation marks).

- Position(i) A number indicating the position of locus i, relative to some arbitray reference point (typically in units of base pairs, but any units can be used: if you use a unit other than base pairs, see the documentation on the -R option). The loci must be in their physical order along the chromosome (ie these Positions must be increasing).

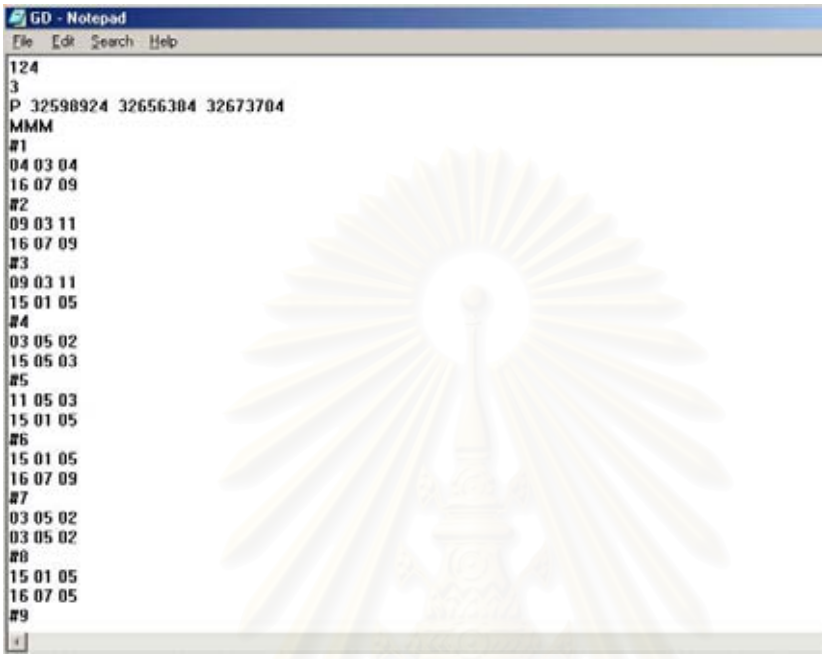
- LocusType(i) A letter indicating the type of locus i. The options are (a) S for a biallelic (SNP) locus, or biallelic site in sequence data. (b) M for microsatellite, or other multi-allelic locus (eg triallelic SNP, or HLA allele). The default assumption is that this denotes a microsatellite locus with stepwise mutation mechanism.

- ID(i) A string, giving a label for individual i.

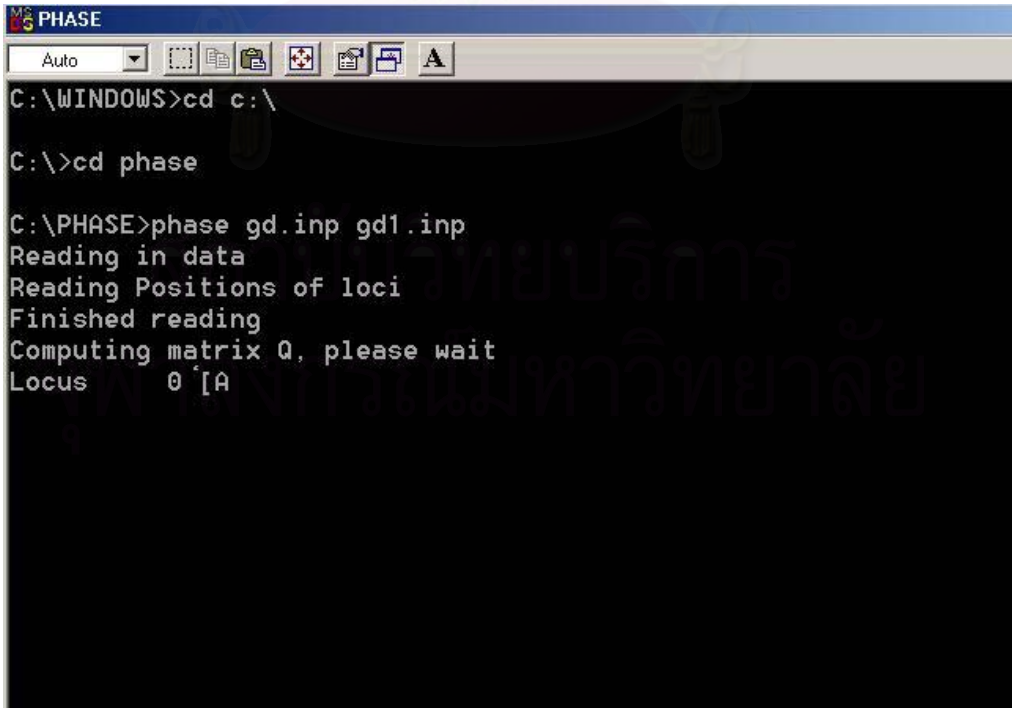
- Genotype(i) The genotypes for the ith individual. This is given on two consecutive rows. At each locus, one allele is entered on the first row, and one on the second row. It does not matter which allele is entered on each row. For biallelic loci, any two characters (e.g. A/C, G/T, 0/1) can be used to represent the two alleles, and they do not need to be separated by a space. Missing alleles at SNP loci should be entered as ?. For multiallelic loci a positive integer must be used for each allele (representing the number of repeats at microsatellite loci), and data for each locus should be separated by aspace. Missing alleles at multiallelic loci should be represented by -1.

This study, consider the example input file, GD.inp, which is as follows:

The example of input file (GD.inp) was shown below.



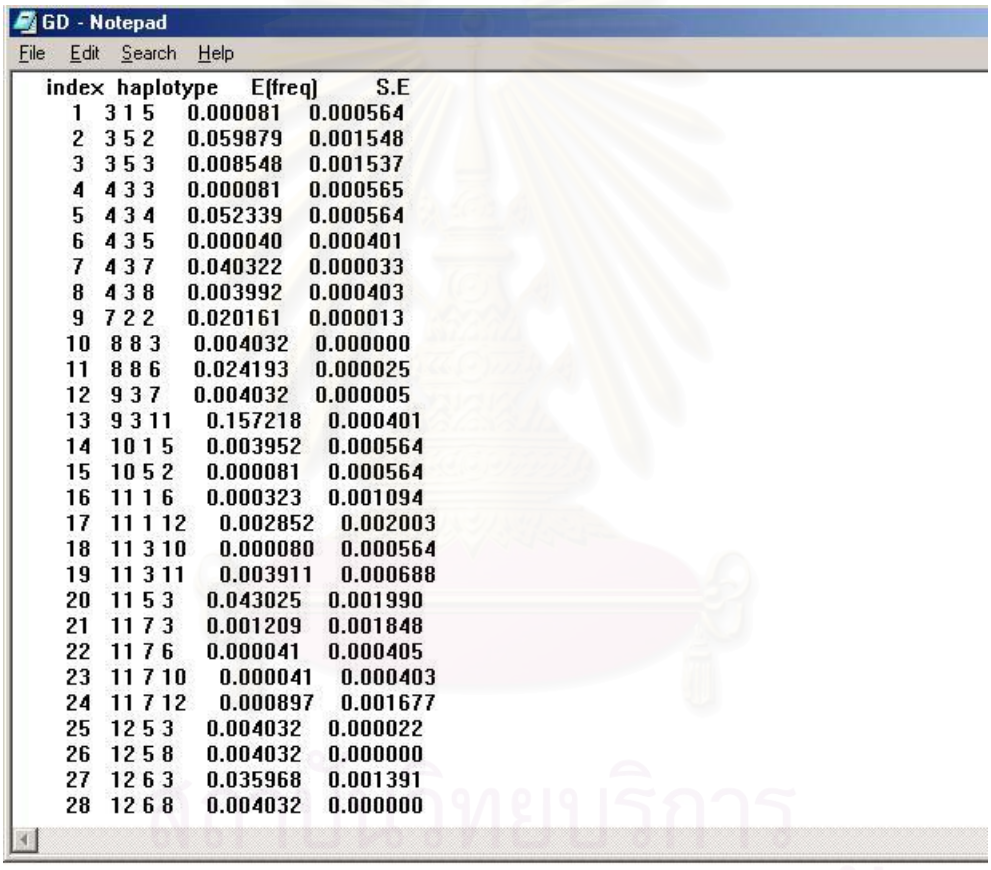
Running of PHASE program was shown below.



Output file

When run, the program initially outputs the data it has read from the input file. The program produces a number of output files. The first, which has the user-specied name, and a similar format to previous versions of PHASE, contains a summary of the individual haplotype estimates for each individual.

The example of output file (GD.inp) was shown below.



BIOGRAPHY

Miss. Thidathip Wongsurawat was born on December 18, 1978 in Nakorn Ratchasima, Thailand. She graduated with the Bachelor degree of Science in Microbiology from Ubon Ratchathani University in 2001 and then attended to particulate in Medical Microbiology program, Graduate School, Chulalongkorn University for her master degree