ความสัมพันธ์ระหว่าง ยีน IL-1Ra, TNF-β, TNF-α, IL-4 และ IFN-γ กับการเกิด Graves' disease ในประชากรไทย

นายจีรวัฒน์ นาคขุนทด

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THE ASSOCIATION BETWEEN IL-1RA GENE, TNF-β GENE, TNF-α GENE, IL-4 GENE AND IFN-γ GENE WITH GRAVES' DISEASE IN THAI

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จีรวัฒน์ นาคขุนทด : ความสัมพันธ์ระหว่าง ยีน IL-1Ra, TNF-β, TNF-α, IL-4 และ IFN-γ กับการเกิด Graves' disease ในประชากรไทย (THE ASSOCIATION BETWEEN IL-1RA GENE, TNF-β GENE, TNF-α GENE, IL-4 GENE AND IFN-γ GENE WITH GRAVES' DISEASE IN THAI) อาจารย์ที่ปรึกษา : ผศ.พญ.ดร. ณัฏฐิยา หิรัญกาญจน์ ; 84 หน้า ISBN 974-17-5625-9

โรคเกรฟเป็นโรคภูมิต้านเนื้อเยื่อของตัวเองที่จำเพาะต่อต่อมไทรอยด์ กระตุ้นให้เกิดการสร้างไทรอยด์ฮอร์โมนจำนวนมาก ปัจจัยที่เป็นสาเหตุของการเกิดโรคเกรฟคือ ปัจจัยทางด้านสิ่งแวดล้อมและปัจจัยทางด้านพันธุกรรม โดยอุบัติการณ์ของโรคเกรฟในแฝดที่เกิดจากไข่ใบเดียวกันสูงกว่าแฝดที่เกิดต่างไข่เป็นข้อบ่งชี้ว่าปัจจัยทางด้านพันธุกรรมมีส่วนสำคัญในการเกิดโรคเกรฟ การศึกษาก่อนหน้านี้รายงานความสัมพันธ์ระหว่างโรคเกรฟกับ human leukocyte antigen และยีน cytotoxic T-lymphocyte antigen-4 แต่ยังไม่มีหลักฐานที่สามารถอธิบายความสัมพันธ์นี้ได้อย่างชัดเจน ดังนั้นยีนไซโตไคน์จึงถูกนำมาศึกษาหาความสัมพันธ์กับการเกิดโรคเกรฟเนื่องจากไซโตไคน์มีความสำคัญในการควบคุมปฏิกริยาตอบสนองทางภูมิคุ้มกันและขบวนการอักเสบ โดยความหลากหลายในยีนไซโตไคน์อาจมีผลต่อกระบวนการ transcription ทำให้การสร้างไซโตไคน์เปลี่ยนแปลงไป งานวิจัยนี้ทำการศึกษาความหลากหลายในยีน IL-1Ra, TNF-α, TNF-β, IL-4 และ IFN-γ ระหว่างผู้ป่วยโรคเกรฟเปรียบเทียบกับคนปกติและศึกษาความสัมพันธ์กับการเกิดโรคเกรฟในคนไทย โดยใช้การศึกษาแบบ population-based case-control รวบรวมผู้ป่วย 137 คนและคนปกติ 137 คนซึ่งมีเชื้อสายและถิ่นกำเนิดเดียวกัน ใช้วิธี PCR และ PCR-SSP หารูปแบบของความหลากหลายในยีน IL-1Ra และ IFN-γ ตามลำดับ วิธี PCR-RFLP หารูปแบบของความหลากหลายในยีน TNF-α, TNF-β และ IL-4 ผลการศึกษาพบว่า –863A allele ของยีน TNF-α ในผู้ป่วยมากกว่าคนปกติอย่างมีนัยสำคัญทางสถิติ (*P*=0.009, OR=1.8, 95% CI=1.15 to 2.84) ซึ่งตำแหน่ง -863 ของยีน TNF-α เกี่ยวข้องกับการจับของ NF-κB ส่วนผลของ –863A allele จะมีลักษณะคล้ายการถ่ายทอดแบบยีนเด่น ซึ่งก็คือต้องการเพียง 1 allele (AC หรือ AA) ในการเพิ่มความเสี่ยง ซึ่งได้ค่าปัจจัยเสี่ยงคือ OR เท่ากับ 2 (*P*=0.01, 95% CI=1.16-3.44) และจากการศึกษาไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของความหลากหลายในยีนไซโตไคน์อื่น ๆ เมื่อเปรียบเทียบระหว่างผู้ป่วยกับคนปกติ ผลสรุปคือ ความหลากหลายที่ตำแหน่ง –863 ในส่วน promoter ของยีน TNF-α สามารถใช้เป็นเครื่องหมายสำหรับยีนที่กำหนดความเสี่ยงในการเกิดโรคเกรฟในคนไทย ซึ่งความเสี่ยงในการเกิดโรคอาจจะเกี่ยวข้องกับการสร้าง TNF-α เพิ่มสูงขึ้น

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

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KEYWORD : GRAVES' DISEASE / CYTOKINE GENE POLYMORPHISMS / IL-1RA GENE / TNF-α GENE / TNF-β GENE / IL-4 GENE / IFN-γ GENE

JEERAWAT NAKKUNTOD : THE ASSOCIATION BETWEEN IL-1RA GENE, TNF-β GENE, TNF-α GENE, IL-4 GENE AND IFN-γ GENE WITH GRAVES' DISEASE IN THAI. THESIS ADVISOR : ASSISTANT PROFESSOR NATTIYA HIRANKARN, MD. Ph.D., 84 pp. ISBN 974-17-5625-9

Graves' disease (GD) is an organ-specific autoimmune disease affecting the thyroid gland, resulting in excessive secretion of thyroid hormone. The etiologic factors identified for GD include both environmental and genetic factors. A genetic contribution to disease was supported by the fact that the incidence of GD in monozygotic twins are much higher than dizygotic twins. Previous studies reported the association between GD with several candidate genes such as human leukocyte antigen and cytotoxic T-lymphocyte antigen-4 gene, but the explanation of this association is still unclear. Cytokine play a key role in the regulation of immune and inflammatory responses and therefore are another potential candidate genes for GD. Since polymorphisms in cytokine genes may effect gene transcription, causing individual variations in cytokine production, the aim of this study was to investigate the polymorphisms of IL-1Ra gene, TNF-α gene, TNF-β gene, IL-4 gene and IFN-γ gene in patients with GD compare with control group and determine the association with GD in Thai population. This population-based case-control study included 137 GD patients and 137 healthy control subjects with similar ethnic and geographic background. IL-1Ra and IFN-γ gene polymorphisms were identified by using polymerase chain reaction (PCR) and PCR-sequence-specific primer (SSP), respectively. TNF-α, TNF-β and IL-4 gene polymorphisms were detected by PCR-restriction fragment length polymorphism (RFLP). As the results, only the –863A allele within TNF-α gene, which has the effect on the binding affinity to NF-κB complex, was found to be significantly increased in GD patients compared to healthy controls (*P*=0.009, OR=1.8, 95% CI=1.15 to 2.84). The effect of –863A allele of TNF-α gene was similar to autosomal dominance mode of inheritance. The presence of one A allele (AA or AC) conferred the significant OR of 2 (*P*=0.01, 95% CI=1.16 to 3.44). There were no statistically significant difference between the study groups for the other cytokine gene polymorphisms. In conclusion, the –863 polymorphism in the promoter region of the TNF-α gene is the marker for genetic susceptibility to GD in Thai population, which may be involved in susceptibility to GD in part through their higher promoter activity of TNF-α production.

Field of study Medical Microbiology Student' s signature……

Academic year 2003 Advisor' s signature……

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ABBREVIATIONS

APCs	antigen-presenting cells
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
IL-1Ra	IL-1 receptor antagonist
IFN	Interferon
kDa	Kilodalton
HLA	Human Leukocyte Antigen
µl	microliter
µg	microgram
ml	milliliter
mM	millimolar
MW	molecular weight
ng	nanogram
NK	Natural Killer
OR	Odd Ratio
PCR	Polymerase Chain Reaction
RFLP	restriction fragment-langth polymorphism
SSP	sequence specific primer
SDS	Sodium Dodecyl sulfate

SNP	Single Nucleotide Polymorphism
TNF	Tumor necrosis factor
TSH	Thyroid-stimulating hormone
U	Unit
VNTR	variable numbers of tandem repeats

CHAPTER I

INTRODUCTION

Graves' disease (GD) is an organ-specific autoimmune disease of the thyroid gland characterized by hyperthyroidism, diffuse goitre, ophthalmopathy and rarely dermopathy (Kita-Furuyama et al., 2003; Ginsberg, 2003). The disease is mediated by autoantibodies that bind to the thyroid-stimulating hormone (TSH) receptor and stimulate thyroid hormone production. These stimulatory antibody belong predominantly to the IgG class and act as TSH agonists (Dogan et al., 2003). GD is common in the general population with a prevalence of 0.5%–1% and a strong female predominance (5-10:1) (Vanderpump and Tumbridge, 1999). GD is a multifactorial disease that develops as the result of a complex interaction between genetic susceptibility genes and environment factors (Davies, 2000). Evidence for the role of genetics factor is show by the indicated incidence of the disease within families and by twin studies. The fact that concordance rates for GD in monozygotic twins, at around 30-40%, are much higher than dizygotic twins supports a genetic contribution to disease (Brix et al., 2001). The lack of a clear pattern of inheritance suggested that multiple genes are involved in influencing the autoimmune events in GD (Farid, 1992). During the last decade, many efforts have been put into the characterization of the genetic background of GD. Several candidate genes have been examined in the past. These have included the human leukocyte antigen (HLA) genes (Heward et al., 1998; Chen et al., 1999; Onuma et al., 1994; Wong et al., 1999), cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene (Yanagawa et al., 1995; Akamizu et al., 2000; Park et al., 2000; Hadj Kacem et al., 2001), vitamin D receptor (Ban and Taniyama, 2000; Pani et al., 2002), Ig heavy chain allotype (Uno et al., 1981), T cell receptor β-chain (Demaine et al., 1987) and TSH receptor (Bohr et al., 1993). However, each of these candidate genes is likely to contribute no more than 5% to the overall genetic susceptibility (Davies, 1998). Other potential susceptibility loci have recently been identified by linkage analysis on chromosomes 14q31 (GD-1),

20q11.2 (GD-2), Xq21 (GD-3), 5q31-q33 and 18q21, but await confirmation (Tomer et al., 1998; Tomer et al., 1998; Tomer et al., 1999; Sakai et al., 2001; Jin et al., 2003; Gough 2000). To understand the mechanisms underlying the development of GD is crucial. Candidate gene studies have proven very effective in detecting susceptibility genes for other diseases as well as genes important for disease progression and is the strategy employed in this study (Roses, 1996; Samson et al., 1996; McDermott et al., 1998).

The role of cytokines in the pathogenesis of GD has been extensively investigated over the past years. In patients with GD, these molecules can be found in both the thyroid and sites of thyroid and sites of extrathyroidal complications of the disease (Ajjan and Weetman, 2003). Cytokines participate in the induction and effector phases of the immune and inflammatory response and are therefore likely to play a critical role in the development of GD (Hunt et al., 2000). Cytokines in the thyroid gland also have a role in regulating antigen presentation and lymphocyte trafficking by enhancing the expression of HLA class II and adhesion molecules on thyroid follicular cells (Ajjan et al., 1996). The production of cytokine varies among individuals and correlates with the polymorphism in the cytokine gene. Polymorphisms within the promoter region may alter binding affinity of transcription factors, such as nuclear factor (NF)-κB, and therefore alter the rate of gene transcription and thus translation resulting in higher protein levels. Polymorphisms within the 5' upstream region and 3' downstream region may alter stability of the transcribed messenger RNA (mRNA) or may alter enhancer activity, and therefore alter the efficiency of gene transcription and mRNA translation. Polymorphisms within introns are less likely to have biological effects yet have still been observed to be associated with biological effect, although this association may frequently be due to linkage with biologically active polymorphisms within other parts of the gene (Holmes et al., 2003).

As cytokines interact functionally, this study was to investigate the polymorphisms of cytokine gene in patients with GD compare with control group and determine the association with GD in Thai population. To investigate whether variability in cytokine genes

may influence disease susceptibility or severity, polymorphisms were assessed in a case control association study using a unified method of genotyping. IL-1 receptor antagonist (IL-1Ra) and IFN-γ gene polymorphisms were identified by using polymerase chain reaction (PCR) and PCR-sequence-specific primer (SSP), respectively. TNF-α, TNF-β and IL-4 gene polymorphisms were detected by PCR-restriction fragment length polymorphism (RFLP). Genotypes and allele frequencies were compared between patients and control subjects.

We hypothesized that specific polymorphisms within IL-1Ra, IFN-γ, TNF-α, TNF-β and IL-4 gene for GD patients might suggest the role of cytokine gene as GD susceptibility gene and might lead to better understanding of mechanism of disease. In addition, this knowledge might lead to the prediction of disease severity, clinical outcome, development of new treatment and prevention. Furthermore, this study also provides the basic knowledge of the frequency of cytokine gene polymorphisms in Thai population.

CHAPTER II

OBJECTIVE

The objective of this study was:

To investigate the polymorphisms of IL-1Ra gene, TNF-β gene, TNF-α gene, IL-4 gene and IFN-γ gene in patients with Graves' disease compare with control group and determine the association with Graves' disease in Thai population.

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 and rarely dermopathy (Kita-Furuyama et al., 2003; Ginsberg, 2003). Robert Graves first identified the association of goiter, palpitation and exophthalmos in 1835, although Caleb Parry had published details of case 10 years earlier (Weetman, 2000). The hyperthyroidism and goiter of GD are caused by stimulation of the thyroid by thyroid-stimulating hormone (TSH) receptor antibodies (Rapoport et al., 1998). These stimulatory antibody belong predominantly to the IgG class and act as TSH agonists (Dogan et al., 2003).

Epidemiology

GD is common in the general population, with a prevalence of 0.5%–1% (Vanderpump and Tumbridge, 1999). Among patients with hyperthyroidism, 60 to 80 percent have GD, depending on regional factors, especially iodine intake (Weetman, 2000). GD is 1/5 to 1/10 as common in men as in women and the risk of onset between the ages of 40 and 60 years (Jacobson et al., 1997). The concordance rate for GD in monozygotic twins is 0.35 compared to 0.03 in dizygotic twins (Brix et al., 2001). The prevalence of GD is similar among whites and Asians, and it is lower among blacks (Vanderpump and Tumbridge, 1999).

Predisposing factors

Susceptibility to GD is determined by a mixture of environmental and genetic factors, which are responsible for the emergence of autoreactivity of T and B cells to the TSH receptor.

Environmental Factors

Female sex is the risk factor for GD suggesting that the modulation of the autoimmune response is caused of estrogen (Weetman, 2000). In some patients, adverse events (such as bereavement, divorce and job loss) precede the onset of GD, supporting the possibility of a role for stress as an initiating factor in the disease by means of neuroendocrine pathways (Chiovato and Pinchera, 1996). A persistent theory on the etiology of autoimmune diseases is that exposure to a particular peptide epitope in an environmental antigen might develop immune reactivity to an amino acid sequence identical to that present in TSH receptor. In 1970s to 1990s many authors reported the molecular mimicry of *Yersinia enterocolitica* membrane antigen to TSH receptor protein (Lidman et al., 1976; Weiss et al., 1983; Shenkman and Bottone, 1976; Wenzel et al., 1990; Wolf et al., 1991). Smoking is weakly associated with Graves' hyperthyroidism and strongly associated with the development of ophthalmopathy (Bartalena et al., 1995).

Genetic Factors

A genetic component to the disease has been suggested owing to familial clustering and twin studies (Stenszky et al., 1985; Davies, 1998; Gough, 2000; Weetman, 2001; Brix et al., 2001). A recent statistical model, based on data from a large twin study, found that 79% of the predisposition to GD is due to genetic factors, with only 21% due to non-genetic (Environmental and Endogenous Factors) influences (Brix et al., 2001).

Stenszky and co-workers initially reported monozygotic twin concordance rates of 30%–40% compared with 3%–9% for dizygotic twins, implicating a genetic aetiology (Stenszky et al., 1985). More recently, Brix and co-workers confirmed these findings in Danish twins, with similar concordance rates (Brix et al., 2001).

The genetic study in GD

Three main approaches have been used in the search for susceptibility loci of GD.

Classical linkage analysis

Linkage is defined as the tendency of genes to be inherited together as a consequence of their physical proximity on a single chromosome. The principle of linkage analysis is based on the fact that if two genes or markers are close together on a chromosome, they will cosegregate because the likelihood that a recombination will occur between them during meiosis is low. Therefore, if a tested marker is close to a disease susceptibility gene, its alleles will cosegregate with the disease in families (Tomer and Davies, 2003). Linkage analysis can be performed as part of genome-wide screening or simply on a single chromosomal region of interest, using a series of microsatellite markers. These markers are generally scattered throughout the genome, and comprise of sequences of 2, 3 or 4 nucleotides repeated a variable number of times. They can be highly polymorphic, with several alleles at a single locus. Microsatellites are usually analysed in data sets of large numbers of families in which DNA is available from two affected siblings, with or without parental DNA. Under a model of Mendelian inheritance, siblings would be expected to share zero alleles 25% of the time, one allele 50% of the time and two alleles 25% of the time. If there is a significant excess of allele sharing between sibpairs with disease, genetic linkage is present (Allahabadia and Gough, 1999). Genetic linkage techniques are powerful tools for analyzing complex disease-related genes because they detect genes that have a major influence on the development of a disease (Greenberg,

1993). However, linkage studies are less sensitive than association studies because they do not detect less influential genes (Greenberg, 1993). A linkage study, therefore, may be negative in the absence of major genes contributing to disease susceptibility.

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 (Allahabadia and Gough, 1999). The association is often expressed as the relative risk (or odds ratio) that an individual will develop the disorder if he or she carries the particular allele or marker, compared to an individual who does not carry the allele or marker (Bidwell et al., 1999). There are at least two possible explanations 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 (LD) with it (Hodge, 1994). 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, it may be easier to recruit adequate numbers of subjects in late-onset diseases such as autoimmune hypothyroidism rather than recruit index cases with additional family members, including parents who are less likely to be alive. Finally, 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 (Allahabadia and Gough, 1999). However, this population-based association method may produce false-positive associations if the patients and controls are not accurately matched (population stratification) (Spielman et al., 1993). Therefore, new association tests have been developed that are family-based and use an internal control group from within each family, thus avoiding the necessity to match patients and controls altogether. The most widely used family-based association test is the transmission disequilibrium test (Tomer and Davies, 2003).

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 susceptibility gene in complex disease (Allahabadia and Gough, 1999). The Transmission Disequilibrium Test (TDT) is a test for linkage in the presence of linkage disequilibrium (Spielman et al., 1993). This procedure evaluates the transmission of alleles from a heterozygous parent to one or more offspring. Under simple Mendelian inheritance; that is, if *T* denotes the number of times that the allele is transmitted, and if *U* denotes the number of times that it is not transmitted, then $(T - U)^2 / (T + U)$ has a χ^2 distribution on 1 , under the null hypothesis of no linkage or association (Dudbridge et al., 2000). Practically, collection of family samples for TDT analysis is easier than collecting affected sibpairs for linkage analysis, as only one affected offspring is needed. This technique, however, may be used with even greater power if two or more affected offspring are available. Hence, the TDT can be used in multiplex families that have already been collected for genome-wide searches and linkage analysis. The main limitation of 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. Until a large number of genes (up to 100000) and their polymorphisms have been identified, genome-wide screens will not be possible using linkage disequilibrium analysis (Allahabadia and Gough, 1999).

Genetic studies in GD

There is a well established association with certain human leukocyte antigen (HLA) alleles that varies among racial groups. In whites populations, HLA-DR3 and HLA-DQA1*0501 are positively associated with GD, whereas HLA-DRB1*0701 protects against it (Heward et al., 1998; Chen et al., 1999). In nonwhite populations, GD has been found to be associated with different HLA alleles. For example, GD has been shown to be associated with HLA-B35, B46, A2 and DPB1*0501 in Japanese (Kawa et al., 1977; Dong et al., 1992;

Onuma et al., 1994); A10, B8 and DQw2 in Indians (Tandon et al., 1990); DR1 and DR3 in South African blacks (Omar et al., 1990); the DRB3*0202/DQA1*0501 haplotype in African Americans (Chen et al., 2000); and B46, DR9, DRB1*303 and DQB1*0303 in Hong Kong Chinese (Cavan et al., 1994; Wong et al., 1999). Genetic polymorphism and in turn structural variants of the HLA molecules on the surfaces of antigen-presenting cells (APCs) may increase susceptibility to GD due to their roles in thymic selection of naive T cells and antigenic peptide presentation to peripheral T cells (Collins and Gough, 2002).

GD is associated with polymorphisms of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene (chromosome 2q33) in several racial groups (Yanagawa et al., 1995; Akamizu et al., 2000; Park et al., 2000; Hadj Kacem et al., 2001). This association may reflect an effect of certain CTLA-4 alleles on the function of autoreactive T cells, because other organ-specific autoimmune disorders are also associated with CTLA-4 polymorphisms. When a CTLA-4 molecule, rather than a CD28 molecule, on a T cell engages CD80 or CD86 co-stimulatory molecules on APCs, the T cell is inactivated.

Other genetic associations has been reported. For example, vitamin D receptor (chromosome 4q) (Ban and Taniyama, 2000; Pani et al., 2002), Ig heavy chain allotype (chromosome 14q32) (Uno et al., 1981) and T cell receptor β-chain (chromosome 7q35) (Demaine et al., 1987). Furthermore, previous studies reported the association between thyroid-specific genes with autoimmune thyroid diseases such as TSH receptor (chromosome 14q31) (Bohr et al., 1993), thyroid peroxidase (chromosome 2p25) (Jaume et al., 1999) and thyroglobulin (chromosome 8q24) (Tomer et al., 2002).

Linkage analysis has identified loci on chromosomes 14q31 (GD-1), 20q11.2 (GD-2), Xq21 (GD-3), 5q31-q33 and 18q21 that are associated with susceptibility to GD (Tomer et al., 1998; Tomer et al., 1998; Tomer et al., 1999; Sakai et al., 2001; Jin et al., 2003; Vaidya et al., 2000) (table 1).

Table 1 Previously reported susceptibility loci with evidence of linkage to GD

Putative candidate gene	Chromosome region	Marker	Ethnic group	Test statistic	Type of study	No. of Families	Studies
CTLA-4	2q33	D2S117	Caucasians (England, Scotland)	NPL=3.43	CLA	64 (ASP)	Vaidya et al., 1999
	5q31-q33	D5S436	Japanese	LOD=3.14	WGS	113 (ASP)	Sakai et al., 2001
	5q31	D5S436	Chinese Han	LOD=2.3	WGS	54 (Ped)	Jin et al., 2003
HLA	6p21	D6S273	Caucasians (England, Scotland)	NPL=1.95	CLA	64 (ASP)	Vaidya et al., 1999
GD-1	14q31	D14S81	Caucasians (U.S., Italy, Israel, UK.)	LOD=2.5	WGS	56 (Ped)	Tomer et al., 1999
IDDM-6	18q21	D18S487	Caucasians (England, Scotland)	NPL=3.46	CLA	67 (ASP)	Vaidya et al., 2000
GD-2	20q11.2	D20S195	Caucasians (U.S., Italy, Israel, UK.)	LOD=3.5	WGS	56 (Ped)	Tomer et al., 1999
GD-2	20q11.2	D20S106	Caucasians (UK)	NPL=2.02	CLA	64 (ASP)	Pearce et al., 1999
GD-3	Xq21.33	DXS8020	Caucasians (U.S., Italy, Israel, UK.)	LOD=2.5	WGS	56 (Ped)	Tomer et al., 1999
IDDMX	Xp11	DXS8083	Caucasians (England, Scotland)	NPL=2.21	CLA	68 (ASP)	Imrie 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

Table 2 Cytokine gene polymorphism and GD

Gene	polymorphism	Ethnic group	Cases/ controls (n)	Odds ratio	*p*	Studies
IL-1Ra	VNTR (intron 2)	English Caucasian	100/261	1.7 (allele 2)	0.024	Blakemore et al., 1995
IL-1Ra	VNTR (intron 2)	North American Caucasian	141/145		NS	Cuddihy and Bahn, 1996
IL-1Ra	VNTR (intron 2)	German	144/174		NS	Muhlberg et al., 1998
IL-1Ra	VNTR (intron 2)	Moscow	78/93		NS	Chistyakov et al., 2000
IL-1Ra	+11100 (T/C) (Exon2)	Caucasian	138/101		NS	Hunt et al., 2000
IL-1α	+4845 (G/T) (Exon 5)	North American Caucasian	141/145		NS	Cuddihy and Bahn, 1996
IL-4	-590 (T/C) (promoter)	Caucasian	138/101	0.4 (T allele)	0.005	Hunt et al., 2000
IL-4	-590 (T/C) (promoter)	Caucasian	381/285		NS	Heward et al., 2001
IFN-γ	CA repeat (intron 1)	Caucasian	202/214		<0.04 (allele 5)	Siegmund et al., 1998
TNF-β	*Nco*I restriction Site (intron 1)	Caucasian	174/173		<0.03 (TNFB*1/2)	Badenhoop et al., 1992
TNF-β	*Nco*I restriction Site (intron 1)	Polish	156/80	2.81 (TNFB*1)	<0.01	Kula et al., 2001
TNF-β	+720, +356, +249	Caucasian	138/267	1.9 (AGG)	$7x10^{-4}$	Hunt et al., 2001
TNF-β	*Nco*I restriction Site (intron 1)	Chinese	57/92		NS	Cavan et al., 1994
TNF-α	-308 (G/A) (promoter)	Polish	156/80	4.38 (A allele)	<0.0001	Kula et al., 2001
TNF-α	+488, -238, -308	Caucasian	138/267	2.2 (GGA)	$3x10^{-5}$	Hunt et al., 2001

NS = not significant

Cytokine gene polymorphism in GD

In 1990s to 2000s many authors reported the cytokine gene polymprphism in GD (table 2). However, the results are still controversy and varied between each ethnic group. For example, Blakemore and co-workers reported an association of GD with an allele of the IL-1 receptor antagonist gene (chromosome 2q14) in English Caucasians (Blakemore et al., 1995), but this was not confirmed by others (Cuddihy and Bahn, 1996; Muhlberg et al., 1998; Heward et al., 1999; Hunt et al., 2000; Chistyakov et al., 2000). Likewise, Hunt and colleagues reported an association of an IL-4 gene (chromosome 5q31) polymorphism with GD in Caucasians (Hunt et al., 2000), whereas Heward and co-workers reported this polymorphism does not play a role in the genetic susceptibility to GD in white Caucasians in the United Kingdom (Heward et al., 2001). Siegmund and co-workers reported an association of GD with an IFN-γ gene (chromosome 12q24.1) polymorphism in Caucasians (Siegmund et al., 1998). Studies in Caucasians have suggested that the TNF-β gene (chromosome 6p21) might be a susceptibility gene for GD (Badenhoop et al., 1992; Hunt et al., 2001; Kula et al., 2001), whereas Cavan and colleagues reported that this polymorphism was not associated with GD in Hong Kong Chinese (Cavan, Penny et al., 1994). Two studies reported an association of GD with TNF-α gene (chromosome 6p21) polymorphism in Polish and Caucasians (Kula et al., 2001; Hunt et al., 2001). Furthermore, Kamizono and co-workers reported an association of TNF-α gene with predispose to the development of ophthalmopathy in Japanese patients with GD (Kamizono et al., 2000).

Cytokine gene polymorphism

The production of cytokine varies among individuals and correlates with the polymorphism in the cytokine gene. Polymorphisms include single nucleotide polymorphisms (SNPs), microsatellite repeats and minisatellite or variable number of tandem repeats (VNTRs). These polymorphisms can be found within the coding regions, introns or promoter regions. When the polymorphisms is within a protein-coding region of a

gene, the variant allele may lead to an amino acid substitution that renders the resulting protein functionally altered (Holmes et al., 2003). Polymorphisms within the promoter region may alter binding affinity of transcription factors, such as NF-κB, and therefore alter the rate of gene transcription and thus translation resulting in higher protein levels. Polymorphisms within the 5' upstream region and 3' downstream region may alter stability of the transcribed messenger RNA (mRNA) or may alter enhancer activity, and therefore alter the efficiency of gene transcription and mRNA translation. Polymorphisms within introns are less likely to have biological effects yet still have been observed to be associated with biological effect. Although this association may frequently be due to linkage with biologically active polymorphisms within other parts of the gene (Holmes et al., 2003). The influence of cytokine gene polymorphisms on gene expression has been addressed at two levels of research. First, *in vitro* gene expression studies. Second, *in vivo* disease association studies.

In vitro gene expression studies

In vitro gene expression studies attempt to determine a genetic basis for interindividual differences in the immune response. This is achieved by examining the relationship between individual polymorphic alleles or haplotypes of cytokine genes and the expression of the transcript or cytokine *in vitro*. The main approaches used to date include measuring the levels of cytokine or cytokine receptor mRNA, or of cytokine or receptor protein, expressed as a result of *in vitro* stimulation of cells in culture with a mitogen; and isolation of individual alleles of gene promoters by cloning adjacent to a reporter gene in an expression vector, followed by transfection of an appropriate cell line and measurement of reporter protein expression (Bidwell et al., 1999).

In vivo gene expression studies

These studies attempt to identify immunogenetic markers for a given disease. Association is sought between specific cytokine gene polymorphisms and clinical outcome by direct comparison of individual cytokine genotypes and the clinical features of the disease (eg, susceptibility, duration and severity). Such data may be generated using population-based or family studies in humans or using animal models, and may be from analysis of secreted, cell surface or intracellular protein, or of cytokine mRNA. Using these and other clues, many studies have identified statistically significant associations between cytokine alleles and disease (Bidwell et al., 1999).

IL-1 receptor antagonist (IL-1Ra)

IL-1 is the product of two polypeptides (IL-1α; 159 amino acids and IL-1β; 153 amino acids), that are encoded by separate genes on chromosome 2 and share only 26% amino acids sequence similarity. IL-1 can be produced by virtually all nucleated cell types, including all members of the monocyte-macrophage lineage, B lymphocyte, natural killer (NK) cells, keratinocytes, dendritic cell, astrocytes, fibroblasts, neutrophils, endothelial cell and smooth muscle cells (Joost et al., 1994). IL-1 possess a wide spectrum of inflammatory, metabolic, physiological, haematopoietic and immunological activities. The IL-1 dependent biological activities require binding of IL-1 to specific cell-surface receptors and normally are balanced by the naturally occurring IL-1Ra, which acts competitively to inhibit binding of IL-1 to its receptors (Dinarello and Thompson, 1991; Arend, 1993). IL-1Ra is a 22 to 25 kDa protein that is related structurally to IL-1α and IL-1β (Blakemore et al., 1995). The originally described isoform of IL-1Ra is secreted from monocytes, macrophages, neutrophils and other cells and is now termed sIL-1Ra. At least three additional intracellular isoforms of IL-1Ra have been described to date. An 18 kDa form of IL-1Ra, created by an alternative transcriptional splice mechanism from an upstream exon,

is called icIL-1Ra1 and is found inside keratinocytes and other epithelial cells, monocytes, tissue macrophages, fibroblasts and endothelial cells (Haskill et al., 1991).

The important role of IL-1 in the orbital immune process in Graves' ophthalmopathy is highlighted by its capacity to stimulate retro-orbital fibroblast proliferation, glycosaminoglycan synthesis and various immunomodulatory molecules expressed by retro-orbital fibroblast (Dinarello, 1996; Bahn and Heufelder, 1992; Korducki et al., 1992; Heufelder and Bahn, 1992). A biological significance for the IL-1ra in Graves' ophthalmopathy have been reported. Tan and co-workers have reported that exogenous addition of recombinant IL-1Ra can inhibit IL-1 induced stimulation of glycosaminoglycan synthesis in cultured human retro-orbital fibroblasts, suggesting that IL-1 mediated effects in Graves' ophthalmopathy may be counteracted by IL-1Ra *in vitro* (Tan et al., 1996). Furthermore, two previous studies have demonstrated that cultured retro-orbital fibroblasts derived from patients with Graves' ophthalmopathy express and release significantly lower quantities of icIL-1Ra and sIL-1Ra compared with normal retro-orbital fibroblasts (Hofbauer et al., 1997; Muhlberg et al., 1997).

IL-1Ra gene polymorphism

The gene for IL-1, IL-1 receptor and IL-1Ra are closely located on the long arm of chromosome 2q12-22 (Niino et al., 2001). The polymorphic region within intron 2 of the IL-1Ra gene contains a variable numbers of tandem repeats (VNTR) of 86 bp; five alleles of the IL-1Ra gene have been reported (allele 1 to 5), corresponding to 4, 2, 3, 5 and 6 copies of the 86-bp sequence, respectively (Tarlow et al., 1993). The most frequent allele was designated as allele 1 (Arnalich et al., 2002). The 86 bp VNTR polymorphism at intron 2 of the IL-1ra gene has three protein-binding sites: an α-interferon silencer A; a β-interferon silencer B; and an acute-phase response element (Tarlow et al., 1993). The variable copy number of repeats may have the functional difference by binding a variable number of regulatory proteins and could affect the transcription of the IL-1ra gene. It was reported

that IL-1Ra plasma levels are coordinately regulated by both IL-1Ra gene and IL-1β genes (Hurme and Santtila, 1998). However, *in vitro* activated peripheral blood mononuclear cells (PBMCs) from healthy IL-1Ra gene allele 2 carriers produce higher levels of IL-1Ra than the noncarriers (Danis et al., 1995). Furthermore, it has been demonstrated that alleles of the IL-1β gene are not major regulators of IL-1β production, but the IL-1Ra allele 2 strongly increased *in vitro* production of this cytokine (Santtila et al., 1998).

Blakemore and colleagues reported an association of GD with the IL-1Ra allele 2 in English Caucasians (Blakemore et al., 1995). However, lack of an association between the IL-1Ra allele 2 with GD have also been reported in North American Caucasian and German (Cuddihy and Bahn, 1996; Muhlberg et al., 1998).

Interleukin-4

IL-4 is a MW 15,000-20,000 glycoprotein produced primarily by activated $CD4^+$ T lymphocytes, mast cells and basophils (Paul, 1991). IL-4 has multiple immune response-modulating functions on a variety of cell types. It induces immunoglobulin E (IgE) production in B lymphocytes and serves as an important regulator of IgG isotype switching (Del Prete et al., 1988; Vitetta et al., 1985). It also regulates the differentiation of precursor T helper cells into those of the Th2 subset that mediate humoral immunity and modulate antibody production (Romagnani, 1995). In addition, IL-4 promotes cytotoxic T cell activity, enhances IL-13 mediated mast cell growth, acts synergistically with Colony-stimulating factor to enhance to growth of various hematopoietic cells and induces the adhesion molecule VCAM-1 on endothelial cells. It also has multiple effects on macrophage. IL-4 can activate macrophage cytocidal functions and increase macrophage expression of class II MHC protein. However, it inhibits the release of proinflammatory cytokine, such as IL-1, IL-6, IL-8 and TNF-α, from activated monocytes (Joost et al., 1994).

In humans, Th1 cytokines (IFN-γ, IL-2 and TNF-β) stimulate the production of an IgG1 isotype response (Abbas et al., 1996), which is the predominant pathogenic TSH receptor autoantibody seen in GD (Weetman et al., 1990; Weetman et al., 1990). IgG1 isotypes are also prevalent among thyroglobulin and peroxidase antibodies (Weetman et al., 1989; Kuppers et al., 1993). Hence, in autoimmune thyroid disease, lower IL-4 activity may result in a propensity to develop IgG1 autoantibodies along with polarizing the immune response toward cell-mediated immunity. Supporting this mechanism, IL-4 has been shown to inhibit organ-specific autoimmune disease in animals (Rapoport et al., 1993; Racke et al., 1994).

IL-4 gene polymorphism

The human IL-4 gene has been mapped to chromosome 5, where it localizes on the long arm on q31-33 and bands together with other cytokine genes including IL-3, IL-5, IL-9, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF). The IL-4 gene is composed of 4 exons and 3 introns, and encodes a mature 129 amino acid glycoprotein (Arai et al., 1989; Banchereau et al., 1994). Rosenwasser and co-workers reported a polymorphism with a C to T exchange at position -590 upstream from the open reading frame of the IL-4 gene, IL-4 -590T, that is associated with increased promoter activity for IL-4 transcription and elevated levels of serum IgE in asthmatic families (Rosenwasser et al., 1995).

Hunt and co-workers suggested that the T allele of the –590C/T polymorphism in the promoter region of the IL-4 gene, or a gene in linkage disequilibrium with this allele, may confer modest protection against the development of GD in Caucasian (Hunt et al., 2000). Whereas, this polymorphism does not play a role in the genetic susceptibility to GD in white Caucasians in the United Kingdom (Heward et al., 2001).

Interferon-γ

In 1957, it was discovered that cells exposed to inactivated viruses produce at least one soluble factor that can interfere with viral replication when applied to newly infected cells. The factor was named Interferons (IFNs) (Isaacs and Lindenmann, 1957). Since the original discovery, the existence of many different IFNs has become evident and they can now be regarded as a family of proteins. IFNs are divided into two groups depending on their biochemical, physiologic and genetic characteristics. Type I IFNs (least 13 IFN-α subtypes, one IFN-β and one IFN-ω) are expressed by both leukocytes and fibroblasts largely as a direct result of viral infection (Ronnblom and Alm, 2002; Adolf, 1995). IFN-α is the primary IFN produced by leukocytes and consists of proteins (MW18,000-20,000) encoded by a family of at least 18 closely related gene (chromosome 9). The amino acid sequences of these various IFN-α proteins are approximately 90% identical to one another. Fibroblasts primarily express IFN-β, a protein that is only about 30% identical to IFN-α, while IFN-ω is approximately 60% and 30% homologous to IFN-α subtypes and IFN-β, respectively (Meager, 1998). Type II family of IFNs is IFN-γ. IFN-γ is a biologically active non-covalently linked homodimer (MW18,000 polypeptides) secreted primarily by NK cells, Th1 $CD4^+$ T-cells and $CD8^+$ T-cells. IFN-γ mediates a wide range of immunomodulatory effects on both innate and acquired immunity. In most cells, including professional antigen presenting cells, IFN-γ upregulates the expression of major histocompatibility complex (MHC) class I and II molecules and antagonizes viral replication. IFN-γ is also a potent coactivator of macrophages and promotes the differentiation of naive $CD4^+$ T lymphocytes into the Th1 subclass. In B cells it promotes the expression of certain Ig isotypes (Boehm et al., 1997).

Intrathyroidal inflammatory cells and thyroid follicular cells have been shown to produce a variety of cytokines, including IFN-γ (Ajjan et al., 1996). IFN-γ mRNA was detected in eye muscle tissue from almost all patients with thyroid-associated

ophthalmopathy (Hiromatsu et al., 2000). IFN-γ stimulate glycosaminoglycan production by retroocular fibroblasts (Smith et al., 1991). IFN-γ induce the expression of HLA-DR and adhesion molecule (such as ICAM-1) on orbital fibroblasts and eye muscle cells (Hiromatsu et al., 1987; Heufelder and Bahn, 1992).

IFN-γ gene polymorphism

The gene for human IFN-γ is located at chromosome 12q24.1 (Pravica et al., 1999). Two previous studies have described a variable length CA repeat sequence in the first intron of the human IFN-γ gene and showed that allele 2 is associated with high *in vitro* IFN-γ production (Pravica et al., 1999; Awad et al., 1999). Recently, Pravaca and co-workers reported an absolute correlation between this 12-CA-repeat allele (allele 2) and the presence of the T allele at a SNP located at the +874 position (+874T/A) from the translation start site, coinciding with a putative NF-κB binding site that might be important in the induction of constitutively high IFN-γ production (Pravica et al., 2000). Therefore, it has been suggested that the T to A polymorphism at +874 (at the 5' end of the CA repeat) directly influences the level of IFN-γ production associated with the CA microsatellite marker (Pravica et al., 2000).

The association between polymorphism of IFN-γ and GD has been reported. In 1998, Siegmund and co-workers reported an association of GD with variable length CA repeat of the human IFN-γ gene in Caucasians (Siegmund et al., 1998).

Tumor necrosis factor

The family of tumor necrosis factor (TNF) comprises three member: TNF-α, TNF-β (also know as lymphotoxin α, LT-α) and LT-β (Eigler et al., 1997). Genes coding for TNF lie within the HLA class III region, located 250 kb centromeric to the HLA-B locus

and 850 kb telomeric to the class II HLA-DR locus on chromosome 6p21.3 (Nishimura et al., 2000). TNF-α and TNF-β are closely related cytokines that share 30% amino acid residues and use the same cell surface receptor (TNFR-I, p55 and TNFR-II, p75) (Beutler and Cerami, 1989; Bazzoni and Beutler, 1996). Both TNF-α and TNF-β are important mediators of B cell proliferation and humoral immune response (Douni et al., 1995).

Tumor necrosis factor-α

TNF-α is a cytokine with a wide range of pro-inflammatory activities (Vassalli, 1992; Beutler, 1995). It is produced predominantly by activated macrophages and less so by other cell types. Human TNF-α is synthesized as a pro-protein comprising 233 amino acids, with a molecular mass of 26 kDa. The pro-protein is cleaved by a specific metalloprotease (also named TNF-α converting enzyme, TACE) to yield a monomeric form of 17 kDa comprising 157 nonglycosylated amino acids. Under physiological conditions, TNF-α forms a noncovalently bound cone-shaped homotrimer (Jones et al., 1989).

TNF-α is an important mediator of inflammatory and immune functions linked to the pathogenesis and maintenance of autoimmune thyroid disease (Weetman and Rees, 1988; Deuss et al., 1992; Pang et al., 1993; Rasmussen et al., 1994). TNF-α and TNF-α mRNA were detectable in thyroid and orbital tissues form patients with autoimmune thyroid disease and thyroid-associated ophthalmopathy using reverse transcriptase polymerase chain reaction (RT-PCR) technology and immunohistochemistry (Heufelder and Bahn, 1993; Aust et al., 1996; Hiromatsu et al., 1996). IFN-γ and TNF-α can induce the expression of adhesion molecules, regulatory molecules and HLA class II molecules on thyroid epithelial cells, allowing these cells to present antigens to activated T cells (Buscema et al., 1989). This presentation of antigen might exacerbate the autoimmune processes involved in the pathogenesis of GD or other autoimmune thyroid diseases (Weetman, 2000). TNF-α is also

secreted by thyroid epithelial cells, fibroblasts and lymphocytes within the thyroid (Ajjan et al., 1996; Aust et al., 1996).

TNF-α gene polymorphism

The human TNF-α gene is located 850 kb telomeric of the class II HLA-DR locus and 250 kb centromeric of the class I HLA-B locus of the short arm of chromosome 6 (Kamizono et al., 2000). Polymorphism in the promoter region of the TNF-α gene may be important for TNF-α gene expression and protein production. Kamizono and colleagues reported an association of TNF-α gene at position –863 (C to A) with predispose to the development of ophthalmopathy in Japanese patients with GD (Kamizono et al., 2000). In 2000, Udalova and co-workers demonstrated a clear effect of this nucleotide change on the relative binding affinities of different forms of the NF-κB complex. It was shown that the p50-p50 homodimeric form of this complex had a significantly decreased affinity to its DNA binding site for -863A. As the p50-p50 homodimer acts as a transcriptional repressor on binding to its regulatory site in the promoter region of the TNF gene, decreased binding is thought to result in an inadequate down-regulation of TNF gene expression, and thus increased TNF-α production (Udalova et al., 2000).

Tumor necrosis factor-β

TNF-β or lymphotoxin α (LT-α) is mature protein of 171 amino acids, with a molecular mass of 25 kDa. TNF-β was described as a cytotoxic factor produced by activated T lymphocytes (Paul and Ruddle, 1988). TNF-β carries out most of the same activities as the structurally and genetically related molecule, TNF-α. TNF-β exerts a wide variety of effects in tissue culture ranging from killing tumor cells, to inducing gene expression and to stimulating fibroblast proliferation (Ruddle, 1992). IFN-γ and TNF-β induces MHC class II expression and enhances class I expression on thyroid cells. Thus,

TNF-β might be also involved functionally in the development of autoimmune thyroid disease (Buscema et al., 1989).

TNF-β gene polymorphism

The human TNF-β gene is tandemly arranged and maps between the MHC class III and class I genes on the short arm of chromosome 6 (Spies et al., 1986; Ragoussis et al., 1988). In the first intron of the TNF-β gene, there is a *Nco*I polymorphism consisting of the allele TNFB*1 in the presence of the restriction site (CCATGG) and the allele TNFB*2 in its absence (Dawkins et al., 1989). TNFB*1 is associated with higher TNF-β and TNF-α production (Messer et al., 1991; Abraham et al., 1993). In addition, TNFB*1 is linked with an amino acid substitition at position 26 (asparagines in the TNFB*1, threonine in the TNFB*2 sequence) of TNF-β (Messer et al., 1991). This single amino acid substitution may further lead to altered biological activity of TNF-β (Yamaguchi et al., 2001).

Badenhoop and co-workers have reported that patients with TSH receptor antibodies positive GD had more heterozygotes for the TNFB*1/2 allele and less homozygotes for the TNFB*2 allele than controls. When they analyzed HLA-DR3 positive patients and controls separately, TNFB*1/2 heterozygotes were still significantly increased in the patients with DR3 positive GD as compared to DR3 negative patients with GD and controls. They suggested that heterozygotes for the TNFB*1/2 allele might represent an immunologic subset of the disease, the TNFB*1/2 allele may represent an additional susceptibility marker in GD in Caucasians (Badenhoop et al., 1992). In Asian, Chung and co-workers found that the antigenic frequency of HLA-DR8 was increased significantly in Korean patients with thyrotropin receptor blocking antibody (TBII) positive atrophic autoimmune thyroiditis and the TNFB*1 allele was significantly linked to HLA-DR8 in Korean patients with TBII positive atrophic autoimmune thyroiditis (Chung et al., 1994). whereas

Cavan and colleagues reported this polymorphism not a susceptibility for GD in Hong Kong Chinese (Cavan et al., 1994).

CHAPTER IV

MATERIALS AND METHODS

Subjects

One hundred and thirty-seven Thai patients with GD attending at King Chulalongkorn Memorial hospital were included in the study. One hundred and thirty-seven 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 3.

DNA extraction

DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as anticoagulant, using a salting out method (Miller 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.

Genotyping methodology

Polymerase chain reaction (PCR) analysis of IL-1Ra

The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the IL-1Ra gene specific primers described by Arnalich and colleagues (Arnalich et al., 2002). Specific primers amplified the 5 alleles VNTR of 86 bp within intron 2 of the IL-1Ra gene (table 4).

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 and 1 μl (20 pmol) of each primer. 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 (59°C, 50 seconds) and extension (72°C, 20 seconds) and final extension at 72°C for 7 minutes. The PCR products of 410 bp (IL-1RN*1, four repeats), 240 bp (IL-1RN*2, two repeat), 500 bp (IL-1RN*3, five repeat), 325 bp (IL-1RN*4, three repeat) and 595 bp (IL-1RN*5, six repeat) were 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) (Arnalich et al., 2002). Negative controls without DNA template were included in each experiment. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Polymerase chain reaction-sequence specific primer (PCR-SSP) analysis of IFN-γ

Polymorphisms at +874T/A within intron 1 of the IFN-γ gene were identified by using the PCR-SSP method. The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the IFN-γ gene specific primers described by Pravica and colleagues (Pravica et al., 2000) (table 4).

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 human growth hormone sequence. 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 95°C for 1 minutes, followed by 10 cycles of denaturation (95°C, 15 seconds), annealing (62°C, 50 seconds) and extension (72°C, 40 seconds) and 20 cycles of denaturation (95°C, 20 seconds), annealing (56°C, 50 seconds) and extension (72°C, 50 seconds) final extension at 72°C for 7 minutes (Pravica et al., 2000). 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 IFN-γ gene and human growth hormone gene showed

band of 261 and 428 bp fragment, respectively. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Additionally, the PCR products were analyzed to confirm the results of IFN-γ genotyping by DNA sequencing. Specific primers for sequencing described by Awad and colleagues (Awad et al., 1999). The reaction volume for the amplification reaction was 50 μl, containing 400 ng/μl genomic DNA, 0.25 μl of 5.0 U Taq polymerase (Promega or Gibco), 5 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM deoxynucletide triphosphates and 2.5 μl (20 pmol) of each primer. The PCR protocol consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation (95°C, 30 seconds), annealing (56°C, 30 seconds) and extension (72°C, 1 minutes) 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. Successful amplification yields a 119 bp fragment. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments. After the process of sequencing, the sequences of each allele were perceived using CHROMAS program.

Polymerase chain reaction-restriction fragment-langth polymorphism (RFLP) analysis of TNF-α, TNF-β and IL-4

PCR-RFLP analysis of TNF-α

Polymorphism at –863C/A in the promoter region of the TNF-α gene were identified by using the PCR-RFLP method. The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the TNF-α gene specific primers described by Wennberg and colleagues (Wennberg et al., 2002) (table 4).

The reaction volume for the amplification reaction was 30 μl, containing 50 ng/μl genomic DNA, 0.15 μl of 5.0 U Taq polymerase (Promega or Gibco), 3 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 1.8 μl of 25 mM $MgCl_2$, 0.6 μl of 10 mM deoxynucletide triphosphates and 1.5 μl (20 pmol) of each primer. 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 30 cycles of denaturation (94°C, 20 seconds), annealing (59°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. Successful amplification yields a 124 bp fragment. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Ten microliters of amplified DNA were digested with 5 U of restriction enzyme *Tai*I (New England Biolabs, Hitchin, UK) in 1X NEBuffer 1 in a total volume of 15 μl at 37°C for 10-14 hours, followed by 3% agarose gel electrophoresis. If an A was present at position –863, the enzyme would cut the 124 bp PCR product into two frangments, 102 and 22 bp. No digestion would occur if a C was present (Wennberg et al., 2002). A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Additionally, the PCR products were analyzed to confirm the results of TNF-α genotyping by DNA sequencing.

PCR-RFLP analysis of TNF-β

The *Nco*I polymorphism in intron 1 of the TNF-β gene was determined by PCR-RFLP method. The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the TNF-β gene specific primers described by Yamaguchi and colleagues (Yamaguchi et al., 2001) (table 4).

The reaction volume for the amplification reaction was 30 μl, containing 50 ng/μl genomic DNA, 0.15 μl of 5.0 U Taq polymerase (Promega or Gibco), 3 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 1.8 μl of 25 mM $MgCl_2$, 0.6 μl of 10 mM deoxynucletide triphosphates and 1.5 μl (20 pmol) of each primer. 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 30 cycles of denaturation (94°C, 20 seconds), annealing (59°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. Successful amplification yields a 289 bp fragment. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Ten microliters of amplified DNA were digested with 5 U of restriction enzyme *Nco*I (New England Biolabs, Hitchin, UK) in 1X NEBuffer 4 in a total volume of 15 μl at 37°C for 10-14 hours, followed by 3% agarose gel electrophoresis. If a G (allele 1) was present in intron 1, the enzyme would cut the 289 bp PCR product into two frangments, 228 and 61 bp. No digestion would occur if an A (allele 2) was present (Yamaguchi et al., 2001). A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

The PCR products were analyzed to confirm the results of TNF-β genotyping by DNA sequencing.

PCR-RFLP analysis of IL-4

Polymorphism at –590C/T in the promoter region of the IL-4 gene were identified by using the PCR-RFLP method. The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the IL-4 gene specific primers described by Michel and colleagues (Michel et al., 2001) (table 4).

The reaction volume for the amplification reaction was 30 μl, containing 50 ng/μl genomic DNA, 0.15 μl of 5.0 U Taq polymerase (Promega or Gibco), 3 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 1.8 μl of 25 mM $MgCl_2$, 0.6 μl of 10 mM deoxynucletide triphosphates and 1.5 μl (20 pmol) of each primer. 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 30 cycles of denaturation (94°C, 20 seconds), annealing (59°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. Successful amplification yields a 252 bp fragment. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Ten microliters of amplified DNA were digested with 1 U of restriction enzyme *BsmF*I (New England Biolabs, Hitchin, UK) in 1X NEBuffer 4 in a total volume of 15 μl at 65°C for 10-14 hours, followed by 3% agarose gel electrophoresis. If a C was present at position

–590, the enzyme would cut the 252 bp PCR product into two frangments, 192 and 60 bp. No digestion would occur if a T was present (Michel et al., 2001). A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

The PCR products were analyzed to confirm the results of IL-4 genotyping by DNA sequencing.

DNA sequencing

DNA sequencing were used to validate the results of cytokine gene polymorphism by PCR-SSP and PCR-RFLP 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.

Statistical analysis

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). Furthermore, the mode of inheritance analysis was also included.

Table 3 Characteristics of healthy controls and patients with GD

Characteristics	healthy controls	GD
Number of patients	137	137
Females/male	100/37	121/16
Mean age ± SD yr	23 ± 12.3	38.6 ± 12.6

Table 4 Primers used for analysis of the IL-1Ra, IFN-γ, TNF-α, TNF-β and IL-4 gene

Gene polymorphism and primer	Sequence (5'→3')	Reference
IL-1Ra (intron 2)		(Arnalich et al., 2002)
Forward	TCCTGGTCTGCAGGTAA	
Reverse	CTCAGCAACACTCCTAT	
IFN-γ (+874T)		(Pravica et al., 2000)
Forward	TTCTTACAACACAAAATCAAATCT	
Forward	TTCTTACAACACAAAATCAAATCA	
Reverse	TCAACAAAGCTGATACTCCA	
Internal control primer		
Forward	GCCTTCCCAACCATTCCCTTA	
Reverse	TCACGGATTTCTGTTGTGTTTC	
Sequencing primer		(Awad et al., 1999)
Forward	GCTGTCATAATAATATTCAGAC	
Reverse	CGAGCTTTAAAAGATAGTTCC	
TNF-α (-863)		(Wennberg et al., 2002)
Forward	GGCTCTGAGGAATGGGTTAC	
Reverse	CTACATGGCCCTGTCTTCGTTACG	
TNF-β (intron 1)		(Yamaguchi et al., 2001)
Forward	AGACGTTCAGGTGGTGTCAT	
Reverse	TCTGACTCTCCATCTGTCAG	
IL-4 (-590)		(Michel et al., 2001)
Forward	ACTAGGCCTCACCTGATACG	
Reverse	GTTGTAATGCAGTCCTCCTG	

CHAPTER V

RESULTS

1. Polymerase chain reaction (PCR) analysis of IL-1Ra

Five allelles VNTR of 86 bp within intron 2 of the IL-1Ra gene were analyzed by difference size of PCR product. Figure 1 illustrates the representative result showing PCR products from homozygous of IL-1RN*1, heterozygous IL-1RN*1/2, homozygous of IL-1RN*2 and heterozygous IL-1RN*1/4 of IL-1Ra gene.

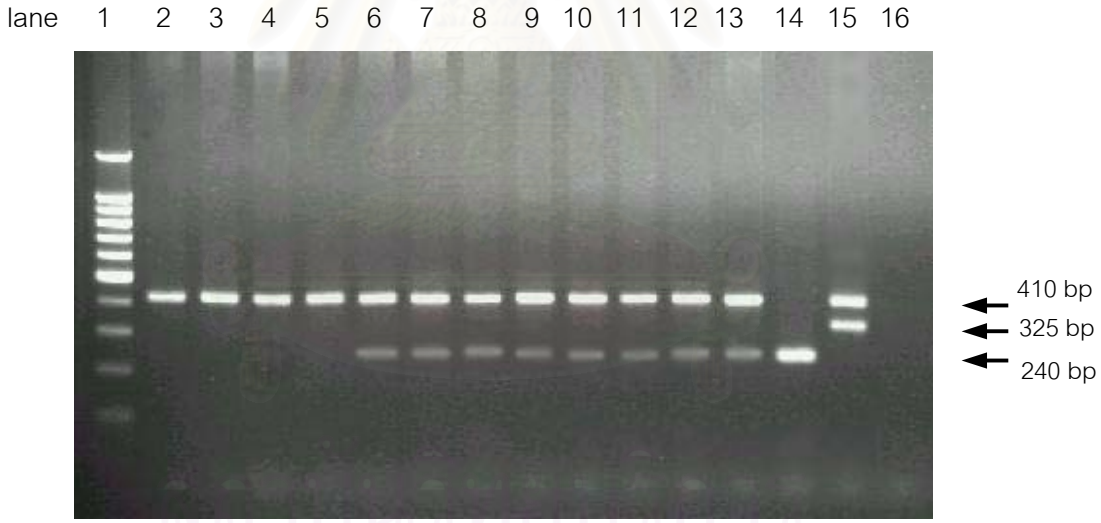


Figure 1 The representative of PCR product from samples with homozygous of IL-1RN*1, heterozygous IL-1RN*1/2, homozygous of IL-1RN*2 and heterozygous IL-1RN*1/4 of IL-1Ra gene. Lane 1 is 100 bp molecular marker, lane 2-5 are homozygous of IL-1RN*1, lane 6-13 are heterozygous IL-1RN*1/2, lane 14 is homozygous of IL-1RN*2, lane 15 is heterozygous IL-1RN*1/4 and lane 16 is Negative control.

2. Polymerase chain reaction-sequence specific primer (PCR-SSP) analysis of IFN-γ

Polymorphisms at +874T/A within intron 1 of the IFN-γ gene were identified by the PCR-SSP method. The positive results of IFN-γ gene and human growth hormone gene (internal control) showed band of 261 and 428 bp fragment, respectively (Figure 2).

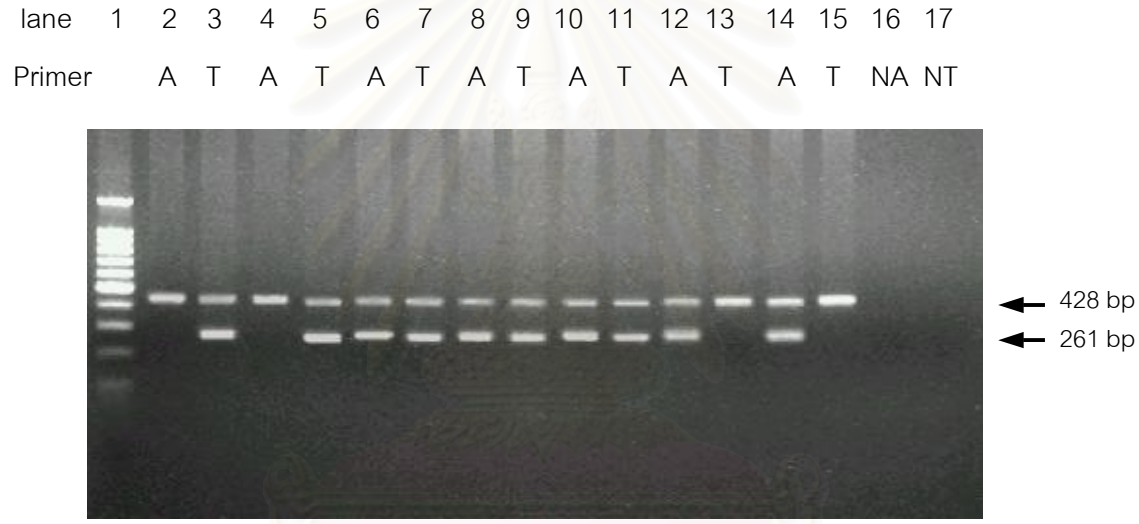


Figure 2 The representative result from samples with homozygous of +874T, heterozygous +874T/A and homozygous of +874A.

Lane 1 is 100 bp molecular marker.

Lane 2-5 (2-3 sample N7 and 4-5 sample N14) are homozygous of +874T.

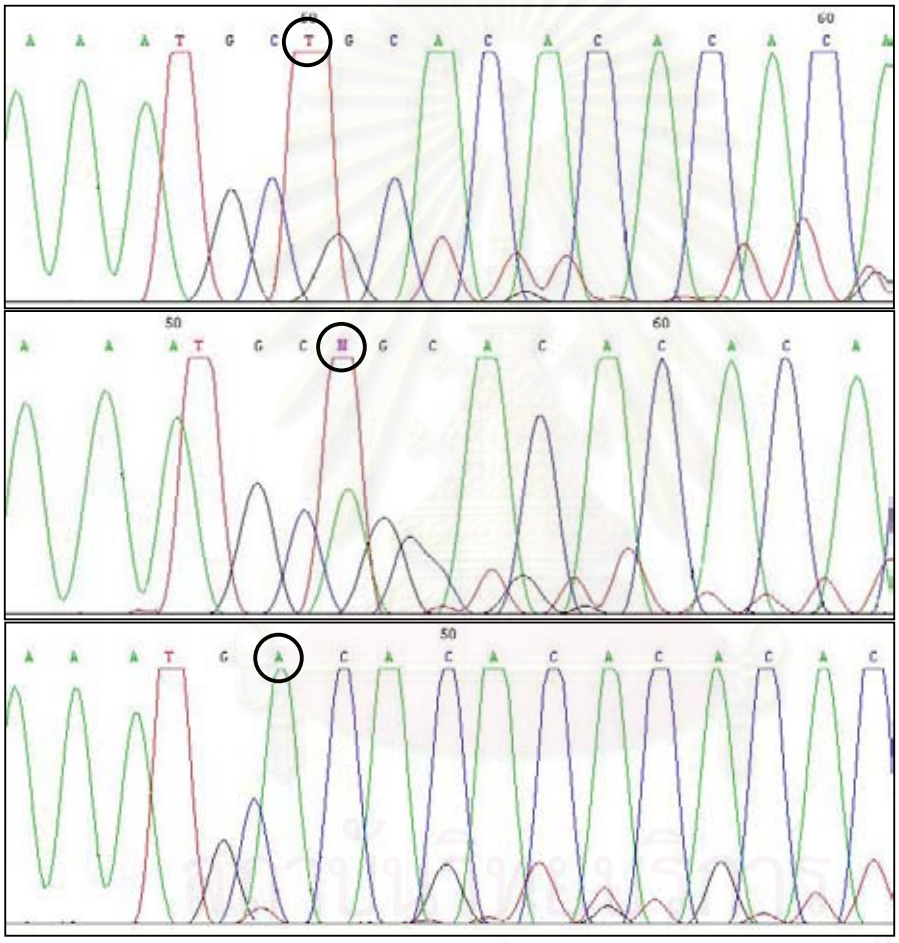
Lane 6-11(6-7sample N9, 8-9 sample N11 and 10-11 sample N17) are heterozygous +874T/A.

Lane 12-15 (12-13 sample N5 and 4-15 sample N6) are homozygous of +874A.

Lane 16-17 are Negative control of in each specific primer (NA is Negative control of +874A allele specific primer and NT is Negative control of +874T allele specific primer).

2.1 Sequencing confirmation of the PCR-SSP results of polymorphisms at +874T/A of the IFN-γ gene

DNA sequencing confirm the results of polymorphisms at +874T/A of the IFN-γ gene from PCR-SSP method as shown in Figure 3.



A

B

C

Figure 3 Chromatogram of DNA sequences in homozygous of +874T, heterozygous +874T/A and homozygous of +874A. Homozygous of +874T from sample N7 (A), heterozygous +874T/A from sample N9 (B) and homozygous of +874A from sample N5 (C).

3. Polymerase chain reaction-restriction fragment-langth polymorphism (RFLP) analysis of TNF-α, TNF-β and IL-4

3.1 PCR-RFLP analysis of TNF-α

Polymorphism at –863C/A in the promoter region of the TNF-α gene were identified by the PCR-RFLP method. If A was present at position –863, the *Tai*I restriction enzyme would cut the 124 bp PCR product into two frangments, 102 and 22 bp. No digestion would occur if C was present (Wennberg et al., 2002) (Figure 4).

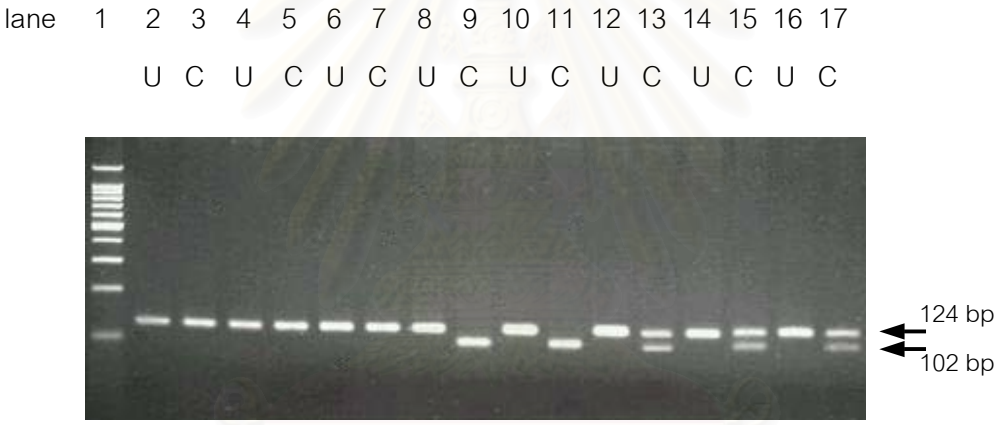


Figure 4 The representative of PCR-RFLP results from samples with homozygous of –863C, homozygous of –863A and heterozygous –863C/A.

Lane 1 is 100 bp molecular marker.

Lane 2-7 (2-3 sample G4, 4-5 sample G7 and 6-7sample G9) are homozygous of -863C.

Lane 8-11(8-9sample G1 and 10-11 sample G5) are homozygous of –863A.

Lane 12-17 (12-13 sample G2, 14-15 sample G6 and 16-17 sample G8) are heterozygous –863C/A. Under these electrophoresis condition the 22 bp product is not visible. U = not add restriction enzyme, C = add restriction enzyme.

3.2 Sequencing confirmation of the PCR-RFLP results of polymorphisms at –863C/A of the TNF-α gene

DNA sequencing confirm the results of polymorphisms at –863C/A of the TNF-α gene from PCR-RFLP method as shown in Figure 5.

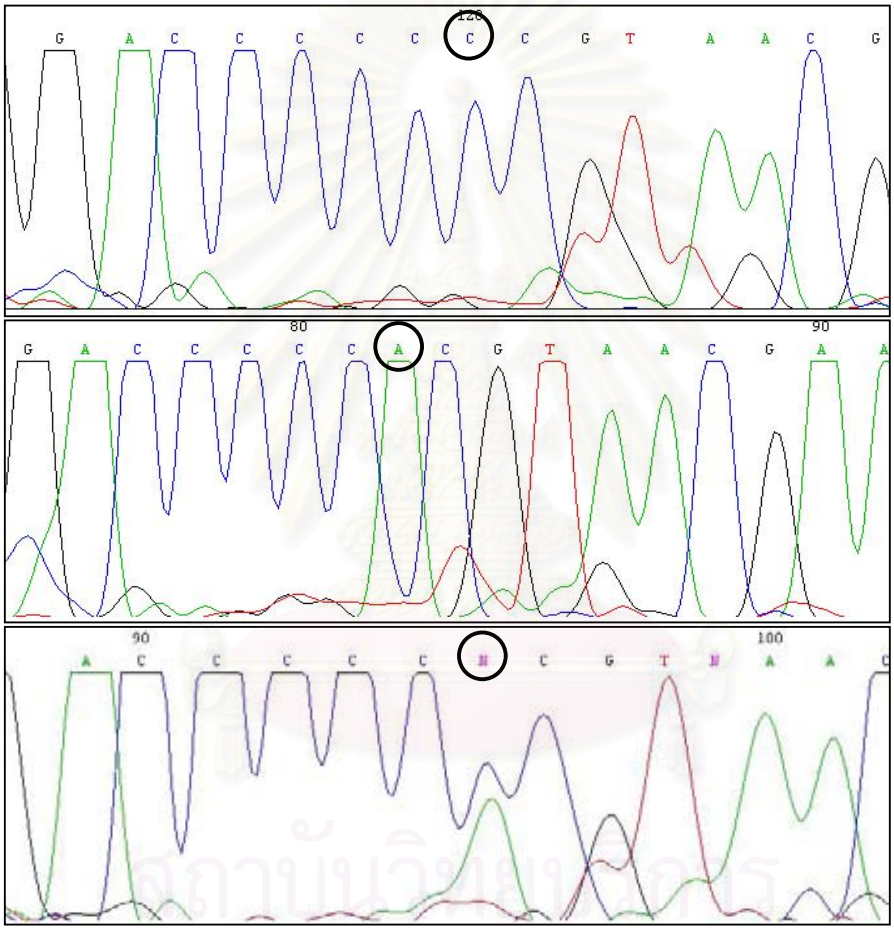


Figure 5 Chromatogram of DNA sequences in homozygous of –863C, homozygous of –863A and heterozygous –863C/A. Homozygous of –863C from sample G4 (A), homozygous of –863A from sample G1 (B) and heterozygous –863C/A from sample G2 (C).

3.3 PCR-RFLP analysis of TNF-β

The *Nco*I polymorphism in intron 1 of the TNF-β gene were determined by PCR-RFLP method. If G (allele 1) was present in intron 1, the *Nco*I restriction enzyme would cut the 289 bp PCR product into two frangments, 228 and 61 bp. No digestion would occur if A (allele 2) was present (Yamaguchi et al., 2001) (Figure 6).

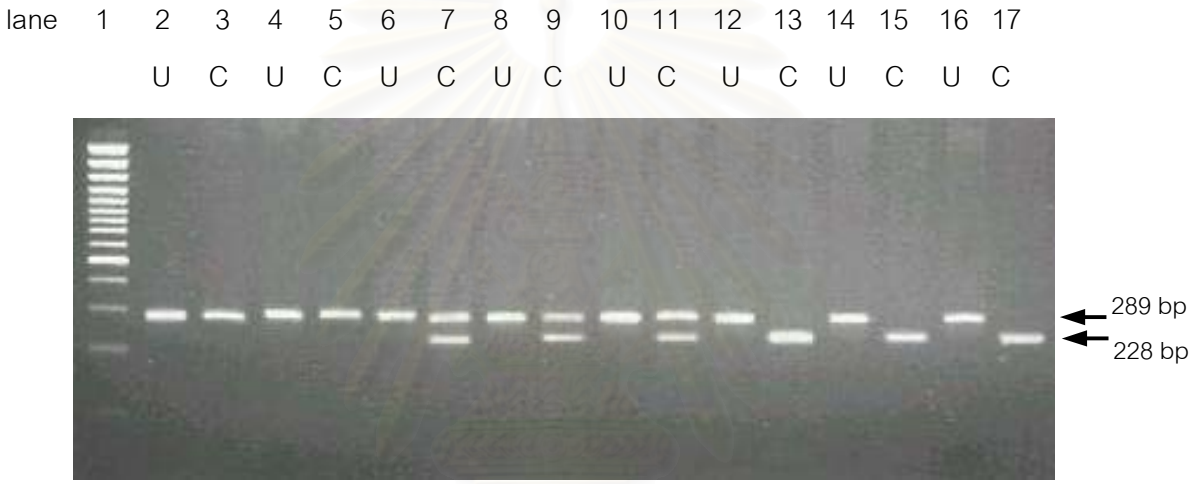


Figure 6 The representative of PCR-RFLP results from samples with homozygous of TNFB*2, heterozygous TNFB*1/2 and homozygous of TNFB*1.

Lane 1 is 100 bp molecular marker.

Lane 2-5 (2-3 sample N3 and 4-5 sample N5) are homozygous of TNFB*2.

Lane 6-11(6-7sample N4, 8-9sample N7 and 10-11 sample N8) are heterozygous TNFB*1/2.

Lane 12-17 (12-13 sample N6, 14-15 sample N12 and 16-17 sample N22) are homozygous of TNFB*1. Under these electrophoresis condition the 61 bp product is not visible. U = not add restriction enzyme, C = add restriction enzyme.

3.4 Sequencing confirmation of the PCR-RFLP results of *Nco*I polymorphism in intron 1 of the TNF-β gene

DNA sequencing confirm the results of *Nco*I polymorphism in intron 1 of the TNF-β gene from PCR-RFLP method as shown in Figure 7.

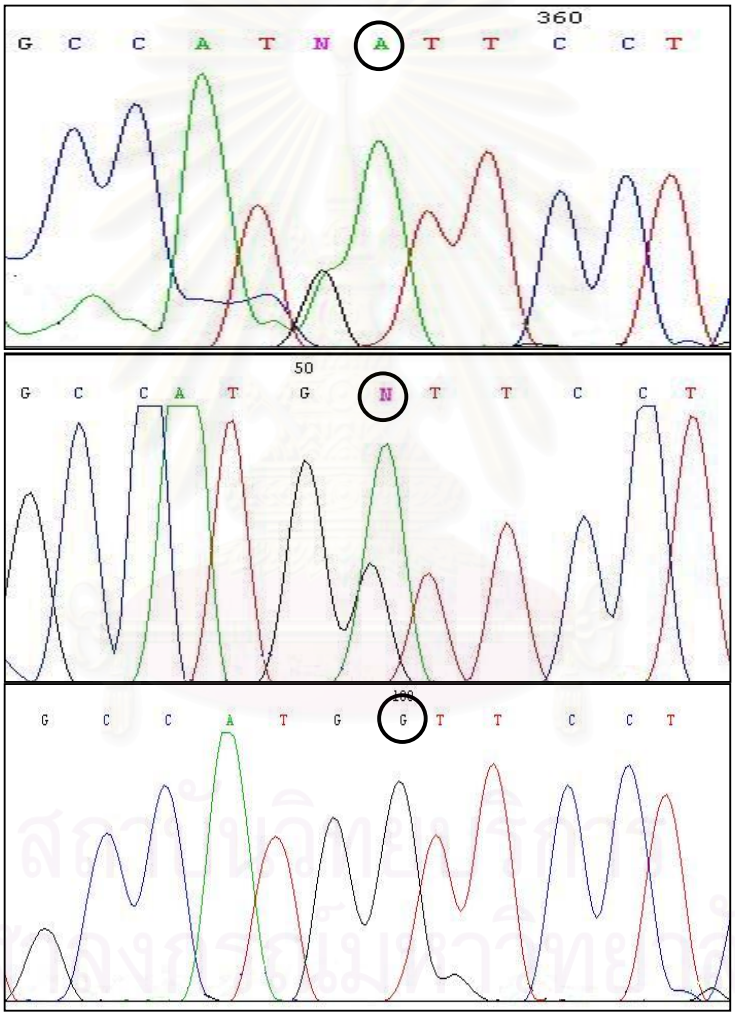


Figure 7 Chromatogram of DNA sequences in homozygous of TNFB*2, heterozygous TNFB*1/2 and homozygous of TNFB*1. Homozygous of TNFB*2 from sample N3 (A), heterozygous of TNFB*1/2 from sample N4 (B) and homozygous TNFB*1 from sample N6 (C).

3.5 PCR-RFLP analysis of IL-4

Polymorphism at –590C/T in the promoter region of the IL-4 gene were identified by the PCR-RFLP method. If C was present at position –590, the enzyme would cut the 252 bp PCR product into two frangments, 192 and 60 bp. No digestion would occur if T was present (Michel et al., 2001) (Figure 8).

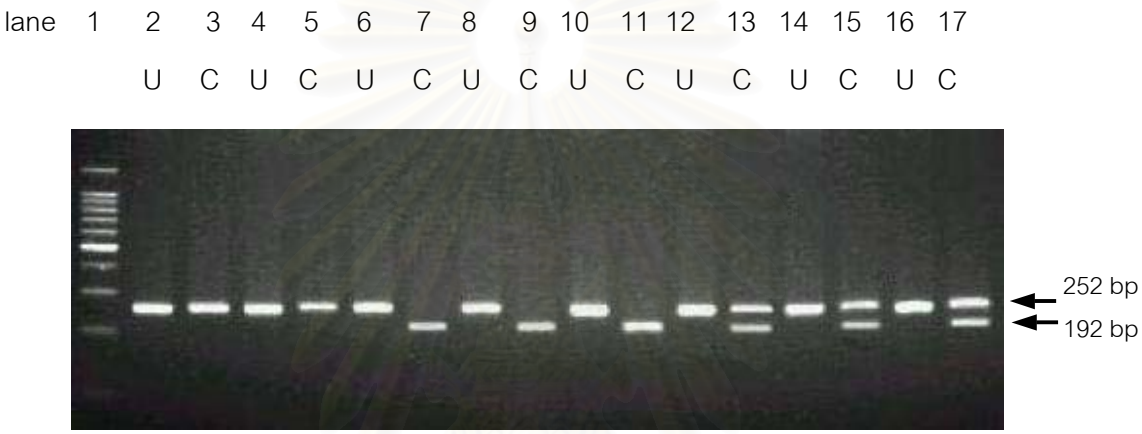


Figure 8 The representative of PCR-RFLP results from samples with homozygous of –590T, homozygous of –590C and heterozygous –590C/T.

Lane 1 is 100 bp molecular marker.

Lane 2-5 (2-3 sample N2 and 4-5 sample N5) are homozygous of –590T.

Lane 6-11(6-7sample N4, 8-9sample N7 and 10-11 sample N33) are homozygous of –590C.

Lane 12-17 (12-13 sample N3, 14-15 sample N6 and 16-17 sample N9) are heterozygous –590C/T. Under these electrophoresis condition the 60 bp product is not visible. U = not add restriction enzyme, C = add restriction enzyme.

3.6 Sequencing confirmation of the PCR-RFLP results of polymorphisms at –590C/T of the IL-4 gene

DNA sequencing confirm the results of polymorphisms at –590C/T of the IL-4 gene from PCR-RFLP method as shown in Figure 9.

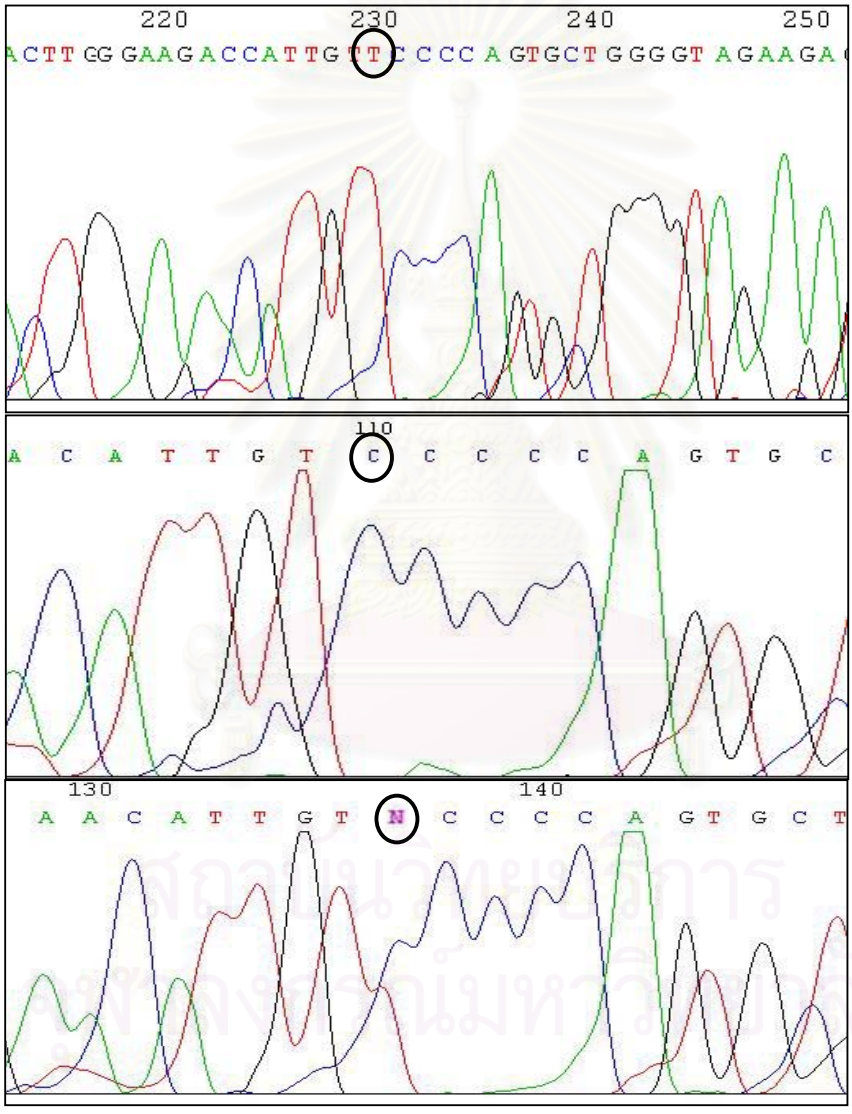


Figure 9 Chromatogram of DNA sequences in homozygous of –590T, homozygous of –590C and heterozygous –590C/T. Homozygous of –590T from sample N2 (A), homozygous of –590C from sample N4 (B) and heterozygous –590C/T from sample N3 (C).

4. The association results of cytokine gene polymorphism with GD

4.1 IL-1ra gene polymorphism

As for the VNTR polymorphisms in intron 2 of IL-1Ra gene, only 1/1, 1/2, 1/4 and 2/2 genotype were detected in both healthy controls and GD patients (table 5 and 6). The vast majority of individuals in this study population carried the 1/1 genotype. One hundred and five of 137 healthy controls (76.6%) were homozygous for the 1/1 genotype, 29 (21.2%) were heterozygous for the 1/2 genotype, 1 (0.7%) was heterozygous for the 1/4 genotype and 2 (1.5%) were homozygous for the 2 genotype. The allele frequencies were 87.6% for 1 allele and 12% for 2 allele. In comparison, 108 of 137 GD patients (78.8%) were homozygous for the 1/1 genotype, 25 (18.3%) were heterozygous for the 1/2 genotype, 1 (0.7%) was heterozygous for the 1/4 genotype and 3 (2.2%) were homozygous for the 2 genotype. The allele frequencies were 88% for 1 allele and 11.3% for 2 allele. There were no significant differences in allele frequency of IL-1Ra VNTR polymorphism between patients with GD and healthy controls.

4.2 IFN-γ gene polymorphism

Genotype and allele frequencies for the +874 at the first intron of IFN-γ gene in healthy controls and GD patients were shown in table 7 and 8. Eighty-three of 137 healthy controls (60.6%) were homozygous for the A/A genotype, 46 (33.6%) were heterozygous and 8 (5.8%) were homozygous for the T/T genotype. The allele frequencies were 77.4% for A allele and 22.6% for T allele. In comparison, 72 of 137 GD patients (52.5%) were homozygous for the A/A genotype, 56 (40.9%) were heterozygous and 9 (6.6%) were homozygous for the T/T genotype. The allele frequencies were 73% for A allele and 27% for T allele. There were no significant differences in allele frequency of +874A/T polymorphism at the first intron of IFN-γ gene between patients with GD and healthy controls.

Table 5 Genotype and allele frequencies of IL-1Ra VNTR polymorphism in healthy controls and GD patients.

	Healthy controls *n* = 137	GD patients *n* = 137
Genotype frequencies		
1/1	105(76.6%)	108(78.8%)
1/2	29(21.2%)	25(18.3%)
1/4	1(0.7%)	1(0.7%)
2/2	2(1.5%)	3(2.2%)
Allele frequencies		
1	240(87.6%)	241(88%)
2	33(12%)	31(11.3%) [a]

[a] *P*=0.91

Table 6 Risk of GD associated with IL-1Ra (VNTR polymorphism) genotype according to different models of inheritance.

	Healthy controls *n* = 137	GD patients *n* = 137
2 dominance, 1 wild type		
1/1	105(76.6%)	108(78.8%)
2/2 or 1/2	31(22.7%)	28(20.5%) [a]
2 recessive, 1 wild type		
1/1 or 1/2	134(97.8%)	133(97.1%)
2/2	2(1.5%)	3(2.2%) [b]

[a] *P*=0.76

[b] *P*=1

Table 7 Genotype and allele frequencies for the +874 at the first intron of IFN-γ gene in healthy controls and GD patients.

	Healthy controls *n* = 137	GD patients *n* = 137
Genotype frequencies		
A/A	83(60.6%)	72(52.5%)
A/T	46(33.6%)	56(40.9%)
T/T	8(5.8%)	9(6.6%)
Allele frequencies		
A	212(77.4%)	200(73%)
T	62(22.6%)	74(27%) [a]

[a] *P*=0.27

Table 8 Risk of GD associated with IFN-γ (+874A/T) genotype according to different models of inheritance.

	Healthy controls *n* = 137	GD patients *n* = 137
T dominance, A wild type		
A/A	83(60.6%)	72(52.6%)
A/T or T/T	54(39.4%)	65(47.4%) [a]
T recessive, A wild type		
A/A or A/T	129(94.2%)	128(93.4%)
T/T	8(5.8%)	9(6.6%) [b]

[a] *P*=0.22

[b] *P*=1

4.3 TNF-α gene polymorphism

Genotype and allele frequencies for the -863 at the promoter of TNF-α gene in healthy controls and GD patients were shown in table 9. Five of 137 healthy controls (3.7%) were homozygous for the A/A genotype, 31 (22.6%) were heterozygous and 101 (73.7%) were homozygous for the C/C genotype. The allele frequencies were 15% for A allele and 85% for C allele. In comparison, 9 of 137 GD patients (6.6%) were homozygous for the A/A genotype, 48 (35%) were heterozygous and 80 (58.4%) were homozygous for the C/C genotype. The allele frequencies were 24% for A allele and 76% for C allele. The –863A allele of TNF-α gene was found to be significantly increased in GD patients compared to healthy controls (*P*=0.009, OR=1.8, 95% CI=1.15 to 2.84).

The effect of –863A allele of TNF-α gene was similar to autosomal dominance mode of inheritance. The presence of one A allele (AA or AC) conferred the significant OR of 2 (*P*=0.01, 95% CI=1.16 to 3.44) (table 10).

4.4 TNF-β gene polymorphism

Genotype and allele frequencies for the *Nco*I restriction site polymorphism at the first intron of TNF-β gene in healthy controls and GD patients were shown in table 11 and 12. Twenty-seven of 137 healthy controls (19.7%) were homozygous for the TNFB*1 genotype, 69 (50.4%) were heterozygous and 41 (29.9%) were homozygous for the TNFB*2 genotype. The allele frequencies were 44.9% for TNFB*1 allele and 55.1% for TNFB*2 allele. In comparison, 29 of 137 GD patients (21.2%) were homozygous for the TNFB*1 genotype, 73 (53.3%) were heterozygous and 35 (25.5%) were homozygous for the TNFB*2 genotype. The allele frequencies were 47.8% for TNFB*1 allele and 52.2% for TNFB*2 allele. There were no significant differences in allele frequency of *Nco*I restriction site polymorphism at the first intron of TNF-β gene between patients with GD and healthy controls.

Table 9 Genotype and allele frequencies for the -863 at the promoter of TNF-α gene in healthy controls and GD patients.

	Healthy controls *n* = 137	GD patients *n* = 137
Genotype frequencies		
A/A	5(3.7%)	9(6.6%)
A/C	31(22.6%)	48(35%)
C/C	101(73.7%)	80(58.4%)
Allele frequencies		
A	41(15%)	66(24%)[a]
C	233(85%)	208(76%)

[a] *P*=0.009, OR=1.8, 95% CI=1.15 to 2.84

Table 10 Risk of GD associated with TNF-α (-863C/A) genotype according to different models of inheritance.

	Healthy controls *n* = 137	GD patients *n* = 137
A dominance, C wild type		
C/C	101(73.7%)	80(58.4%)
A/A or A/C	36(26.3%)	57(41.6%)[a]
A recessive, C wild type		
C/C or A/C	132(96.4%)	128(93.4%)
A/A	5(3.6%)	9(6.6%)[b]

[a] *P*=0.01, OR=2, 95% CI=1.16 to 3.44

[b] *P*=0.41

Table 11 Genotype and allele frequencies for the *Nco*I restriction site polymorphism at the first intron of TNF-β gene in healthy controls and GD patients.

	Healthy controls *n* = 137	GD patients *n* = 137
Genotype frequencies		
1/1(GG)	27(19.7%)	29(21.2%)
1/2(GA)	69(50.4%)	73(53.3%)
2/2(AA)	41(29.9%)	35(25.5%)
Allele frequencies		
1(G)	123(44.9%)	131(47.8%) [a]
2(A)	151(55.1%)	143(52.2%)

[a] *P*=0.54

Table 12 Risk of GD associated with TNF-β (TNFB*1/2) genotype according to different models of inheritance.

	Healthy controls *n* = 137	GD patients *n* = 137
1(G) dominance, 2(A) wild type		
2/2	41(29.9%)	35(25.5%)
1/1 or 1/2	96(70.1%)	102(74.5%) [a]
1(G) recessive, 2(A) wild type		
2/2 or 1/2	110(80.3%)	108(78.8%)
1/1	27(19.7%)	29(21.2%) [b]

[a] *P*=0.49

[b] *P*=0.88

4.5 IL-4 gene polymorphism

Genotype and allele frequencies for the -590 at the promoter of IL-4 gene in healthy controls and GD patients were shown in table 13 and 14. Seventeen of 137 healthy controls (12.4%) were homozygous for the C/C genotype, 50 (36.5%) were heterozygous and 70 (51.1%) were homozygous for the T/T genotype. The allele frequencies were 30.7% for C allele and 69.3% for T allele. In comparison, 15 of 137 GD patients (10.9%) were homozygous for the C/C genotype, 56 (40.9%) were heterozygous and 66 (48.2%) were homozygous for the T/T genotype. The allele frequencies were 31.4% for C allele and 68.6% for T allele. There were no significant differences in allele frequency of –590C/T polymorphism at the promoter of IL-4 gene between patients with GD and healthy controls.

Table 13 Genotype and allele frequencies for the -590 at the promoter of IL-4 gene in healthy controls and GD patients.

	Healthy controls *n* = 137	GD patients *n* = 137
Genotype frequencies		
C/C	17(12.4%)	15(10.9%)
C/T	50(36.5%)	55(40.2%)
T/T	70(51.1%)	67(48.9%)
Allele frequencies		
C	84(30.7%)	85(31%) [a]
T	190(69.3%)	189(69%)

[a] *P*=1

Table 14 Risk of GD associated with IL-4 (-590C/T) genotype according to different models of inheritance.

	Healthy controls *n* = 137	GD patients *n* = 137
C dominance, T wild type		
T/T	70(51.1%)	67(49%)
C/T or C/C	67(48.9%)	70(51%) [a]
C recessive, T wild type		
T/T or C/T	120(87.6%)	122(89%)
C/C	17(12.4%)	15(11%) [b]

[a] *P*=0.81

[b] *P*=0.85

5. Pattern of cytokine gene polymorphism in various populations

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

5.1 Pattern of IL-1ra gene polymorphism

The analysis of 5 allelles of VNTR of 86 bp within intron 2 of the IL-1Ra gene showed significant difference between allele frequencies in Thai population with North American Caucasian (χ^2 =5.4, p=0.02) and Germanian (χ^2 =5.8, p=0.01). There were no significant differences in allele frequencies between Thai population with Ghanaian, Japanese and Taiwanese (table 15).

5.2 Pattern of IFN-γ gene polymorphism

Allele and genotype frequencies for the +874T/A at the first intron of IFN-γ gene were analyzed. There were significant differences in allele and genotype frequencies between Thai population with Italian (χ^2 =9.8, p=0.001 and χ^2 =20.9, p=0.00002, respectively), and Brazilian Caucasian (χ^2 =4.6, p=0.03 and χ^2 =12, p=0.002, respectively). There were no significant differences in allele and genotype frequencies between Thai population with South African and Asian (table 16).

5.3 Pattern of TNF-α gene polymorphism

The analysis polymorphism at –863C/A in the promoter region of the TNF-α gene showed no significant differences in allele and genotype frequencies between Thai population with Swedish, Caucasians, Japanese, Korean and Chinese-Singaporeans (table 17).

5.4 Pattern of TNF-β gene polymorphism

Allele and genotype frequencies for the *Nco*I polymorphism in intron 1 of TNF-β gene were analyzed. There were significant differences in allele and genotype frequencies between Thai population with London, Berlin, Mannheim (χ^2 =4.2, p=0.04 and χ^2 =8.2, p=0.01, respectively), and Spanish (χ^2 =9.8, p=0.001 and χ^2 =20.7, p=0.00003, respectively). There were no significant differences in allele and genotype frequencies between Thai population with Japanese and Korean (table 18).

5.5 Pattern of IL-4 gene polymorphism

Allele and genotype frequencies for the polymorphism at –590C/T in the promoter region of the IL-4 gene were analyzed. The analysis showed significant differences in allele and genotype frequencies between Thai population with Caucasians (χ^2 =65, p<0.05 and χ^2 =98.7, p<0.05, respectively), and Brazilian (χ^2 =19.2, p=0.00001 and χ^2 =39.6, p<0.05, respectively). However, no statistical differences were observed when comparing Thai population with Japanese and Kuwaiti Arab (table 19).

Table 15 Comparison between genotype and allele frequencies of IL-1Ra gene polymorphism in the different population

Cytokine	Position	Genotypes		control					
			Author	Cuddihy and Bahn	Muhlberg et al.	Gyan et al.	Niino et al.	Fuu-Jen Tsai et al.	
			Year	1996	1998	2002	2001	2002	
			Ethnic group	North American Caucasian[a]	Germanian[b]	Ghanaian	Japanese	Taiwanese	Thai
			N	145	174	111	104	83	137
IL-1Ra	VNTR	1/1		75(51.7%)	100(57.5%)	102(91.9%)	87(83.7%)	69(83.1%)	105(76.6%)
	(intron 2)	1/2		48(33.1%)	47(27%)	7(6.3%)	14(13.5%)	14(16.9%)	29(21.2%)
		1/3		6(4.1%)	4(2.3%)	0	2(1.9%)	0	0
		1/4		2(1.4%)	0	0	1(1.0%)	0	1(0.7%)
		2/2		11(7.6%)	21(12.1%)	2(1.8%)	0	0	2(1.5%)
		2/3		3(2.1%)	2(1.1%)	0	0	0	0
		Allele							
		1		206(71%)	251(72.1%)	211(95%)	191(91.8%)	152(91.6%)	240(87.6%)
		2		73(25.2%)	91(26.1%)	11(5%)	14(6.7%)	14(8.4)	33(12%)

[a] χ^2 =5.4, p=0.02 ; compare between allele frequencies in Thai with North American Caucasian

[b] χ^2 =5.8, p=0.01 ; compare between allele frequencies in Thai with Germanian

Not significant ; compare between allele frequencies in Thai with Ghanaian, Japanese and Taiwanese

Table 16 Comparison between genotype and allele frequencies of IFN-γ gene polymorphism in the different population

Cytokine	Position	Genotypes		control				
			Author	Poli et al.	Daher et al.	Govan et al.	Hoffmann et al.	
			Year	2002	2003	2003	2002	
			Ethnic group	Italian [a, c]	Brazilian Caucasian [b, d]	South African	Asian	Thai
			N	363	104	140	29	137
IFN-γ	+874	A/A		32%	39(38%)	102(73%)	19(66%)	83(60.6%)
	(intron 1)	T/A		46.8%	50(48%)	31(22%)	7(24%)	46(33.6%)
		T/T		21.2%	15(14%)	7(5%)	3(10%)	8(5.8%)
		Allele						
		A		55.3%	128(61.5%)	235(84%)	45(77.6%)	212(77.4%)
		T		44.7%	80(38.5%)	45(16%)	13(22.4%)	62(22.6%)

[a] χ^2 =9.8 , p=0.001 ; compare between allele frequencies in Thai with Italian

[b] χ^2 =4.6 , p=0.03 ; compare between allele frequencies in Thai with Brazilian Caucasian

[c] χ^2 =20.9 , p=0.00002 ; compare between genotype frequencies in Thai with Italian

[d] χ^2 =12 , p=0.002 ; compare between genotype frequencies in Thai with Brazilian Caucasian

Not significant ; compare between genotype and allele frequencies in Thai with South African and Asian

Table 17 Comparison between genotype and allele frequencies of TNF-α gene polymorphism in the different population

Cytokine	Position	Genotypes		control					
			Author	Skoog et al.	Wennberg et al.	Kamizono et al.	Park et al.	Ene-Choo Tan et al.	
			Year	1999	2002	2000	2002	2003	
			Ethnic group	Swedish	Caucasians	Japanese	Korean	Chinese-Singaporeans	Thai
			N	156	97	575	190	152	137
TNF-α	-863	A/A		6(3.8%)	2(2%)	10(1.7%)	6(3.2%)	2(1%)	5(3.7%)
	(promoter)	A/C		44(28.2%)	23(24%)	141(24.5%)	51(26.8%)	45(30%)	31(22.6%)
		C/C		106(68%)	72(74%)	424(73.7%)	133(70.0%)	105(69%)	101(73.7%)
		Allele							
		A		56(18%)	27(14%)	161(14%)	63(16.6%)	49(16%)	41(15%)
		C		256(82%)	167(86%)	989(86%)	317(83.4%)	255(84%)	233(85%)

Not significant ; compare between genotype and allele frequencies in Thai with Swedish, Caucasians, Japanese, Korean and Chinese- Singaporeans

Table 18 Comparison between genotype and allele frequencies of TNF-β gene polymorphism in the different population

Cytokine	Position	Genotypes		control				
			Author	Badenhoop et al.	Garrote et al.	Nishimura et al.	Tae-Youn Jun et al.	
			Year	1992	2002	2000	2003	
			Ethnic group	London, Berlin, Mannheim[a, c]	Spanish[b, d]	Japanese	Korean	Thai
			N	173	64	111	202	137
TNF-β	*Nco*I restriction	1/1(G/G)		17(10%)	3(4.7%)	16(14.4%)	44(21.8%)	27(19.7%)
	Site (intron 1)	1/2(G/A)		72(42%)	23(35.9%)	61(55%)	95(47%)	69(50.4%)
		2/2(A/A)		84(48%)	38(59.4%)	34(30.6%)	63(31.2%)	41(29.9%)
		Allele						
		1(G)		106(30.6%)	29(22.7%)	93(41.9%)	183(45.3%)	123(44.9%)
		2(A)		240(69.4%)	99(77.3%)	129(58.1%)	221(54.7%)	151(55.1%)

[a] χ^2 =4.2 , p=0.04 ; compare between allele frequencies in Thai with London, Berlin, Mannheim

[b] χ^2 =9.8 , p=0.001 ; compare between allele frequencies in Thai with Spanish

[c] χ^2 =8.2 , p=0.01 ; compare between genotype frequencies in Thai with London, Berlin, Mannheim

[d] χ^2 =20.7 , p=0.00003 ; compare between genotype frequencies in Thai with Spanish

Not significant ; compare between genotype and allele frequencies in Thai with Japanese and Korean

Table 19 Comparison between genotype and allele frequencies of IL-4 gene polymorphism in the different population

Cytokine	Position	Genotypes		control				
			Author	Heward et al.	Scarel- Caminoga et al.	Noguchi et al.	Hijazi and Haider	
			Year	2001	2002	1998	2000	
			Ethnic group	Caucasian[a, c]	Brazilian[b, d]	Japanese	Kuwaiti Arab	Thai
			N	285	114	215	47	137
IL-4	-590	C/C		222(77.9%)	43(37.7%)	17(7.9%)	3(6.4%)	17(12.4%)
	(promoter)	C/T		58(20.3%)	57(50%)	97(45.1%)	17(36.2%)	50(36.5%)
		T/T		5(1.8%)	14(12.3%)	101(47%)	27(57.4%)	70(51.1%)
		Allele						
		C		502(88%)	143(62.7%)	129(30%)	23(24.5%)	84(30.7%)
		T		68(12%)	85(37.3%)	301(70%)	71(75.5%)	190(69.3%)

[a] χ^2 =65 , p<0.05 ; compare between allele frequencies in Thai with Caucasian

[b] χ^2 =19.2 , p=0.00001 ; compare between allele frequencies in Thai with Brazilian

[c] χ^2 =98.7 , p<0.05 ; compare between genotype frequencies in Thai with Caucasian

[d] χ^2 =39.6 , p<0.05 ; compare between genotype frequencies in Thai with Brazilian

Not significant ; compare between genotype and allele frequencies in Thai with Japanese and Kuwaiti Arab

CHAPTER VI

DISCUSSION

This study was designed to analyze the potential association between cytokine gene polymorphism and GD in Thai population. Out of 5 cytokine genes (IL-1Ra, IFN-γ, TNF-β, TNF-α, IL-4) analyzed in this study, only the –863(C/A) polymorphism in the TNF-α gene promoter was proved to be a marker for susceptibility to GD in Thai population. The –863A allele was found to be significantly increased in GD patients compared to healthy controls (p=0.009, OR=1.8, 95% CI=1.15 to 2.84). This polymorphism has been associated with predispose to the development of ophthalmopathy in Japanese patients with GD (Kamizono et al., 2000). Two previous studies also reported an association of GD with other polymorphic markers within TNF-α gene in Polish (–308A) and Caucasians (+488G, -238G, -308A) (Kula et al., 2001; Hunt et al., 2001).

Several polymorphisms in the promoter region of the TNF-α gene have been proposed to be important for TNF-α gene expression and protein production. However, the results are still controversy and varied between each different studies. Gene reporter assays have been employed to investigate the functional polymorphisms in the promoter region of the TNF-α gene. There are many variables affecting the results of this type of experiments; including the length of the promoter sequence used, the presence or absence of the 3' UTR, the cell type used for transfection, and whether it is of human or nonhuman origin. Different studies have used different approaches, thus making it difficult to draw a general conclusion.

Functional studies of the TNF polymorphism at position -863 (C/A) has been reported. The transcriptional activity by luciferase assay of the –863A polymorphism in response to ConA was two times greater than that of the –863C allele. Morever, TNF-α production by peripheral blood mononuclear cells in response to ConA from donors

possessing these –863A allele were about 1.8 times greater than the levels from donors with the –863C allele (Higuchi et al., 1998). In 2000, Udalova and co-workers demonstrated a clear effect of this nucleotide change on the relative binding affinities of different forms of the NF-κB complex. It was shown that the p50-p50 homodimeric form of this complex had a significantly decreased affinity to its DNA binding site for -863A. As the p50-p50 homodimer acts as a transcriptional repressor on binding to its regulatory site in the promoter region of the TNF gene, decreased binding is thought to result in an inadequate down-regulation of TNF gene expression, and thus increased TNF-α production (Udalova et al., 2000). These result suggest that the –863A allele may be involved in susceptibility to GD in part through their higher promoter activity of TNF-α production. Indeed, the presence of TNF-α has been reported in thyroid tissues form patients with GD using RT-PCR technology and immunohistochemistry (Heufelder and Bahn, 1993; Aust et al., 1996; Hiromatsu et al., 1996). TNF-α is also secreted by thyroid epithelial cells, fibroblasts and lymphocytes within the thyroid (Ajjan et al., 1996; Aust et al., 1996). TNF-α can induce the expression of adhesion molecules, regulatory molecules and HLA class II molecules on thyroid epithelial cells, allowing these cells to present antigens to activated T cells (Buscema et al., 1989). This presentation of antigen might exacerbate the autoimmune processes involved in the pathogenesis of GD or other autoimmune thyroid diseases (Weetman, 2000). As a further step, research in to associations between cytokine genotypes and disease severity, may help both to explain basic biological events and indicate to clinicians ways of predicting, preventing or managing harmful situations in disease with immunological components. Furthermore, further studies are necessary to clarify the exact mechanism of how this TNF-α gene polymorphism results in increased susceptibility to GD.

Although the above explanation supports a pathophysiological mechanism for the association of this TNF-α variant with GD, it is also possible that this association is not due to the TNF-α gene, but to another gene in linkage disequilibrium. The human TNF-α gene is located right within the class III region of the major histocompatibility complex (MHC) on the short arm of chromosome 6 (Kamizono et al., 2000). Other

genes crucial for the immune response and immunoregulatory including MHC, complement components (C2, C4, factor B), lymphotoxin (LTα and β), heat shock proteins (HSP), transporters associated with antigen processing (TAP), LMP, MIC, DMA and DMB are all in close proximity.

The other cytokine gene polymorphisms evaluated in this study did not reveal any significant associations with GD, demonstrating that the VNTR in intron 2 of IL-1Ra gene, SNP at +874 within intron 1 of the IFN-γ gene, *Nco*I restriction site polymorphisms in intron 1 of the TNF-β gene and SNP at –590 in the promoter of the IL-4 gene are not a polymorphic markers for genetic susceptibility of GD in Thai population. However, there are two major possible explanations for these negative results of the cytokine gene polymorphisms with GD in Thai population. First, the negative results in this study does not rule out the importance of that particular cytokine gene. Since we only analyze 1 position within the gene, other polymorphic markers in this cytokine gene might play a role in the genetic susceptibility of GD in Thai population. Therefore, further study of haplotype analysis is necessary. Second, several studies have reported the cytokine gene polymorphism in GD. However, the results are still controversy and varied between each ethnic group. These observations might confer false-positive results. Although case-control study is a sensitive method and is more likely to detect genes of the modest effect, this strategy is prone to inconsistent results due to false positive that may arise from population stratification, which are also more likely to occur in small data set. This problem can be overcome by using family-based association study (Allahabadia and Gough, 1999).

Furthermore, this results report the distribution of the allele and genotype frequencies for VNTR in intron 2 of IL-1Ra gene, SNP at +874 within intron 1 of the IFN-γ gene, *Nco*I restriction site polymorphisms in intron 1 of the TNF-β gene and SNP at –590 in the promoter of the IL-4 gene in Thai population. In summary, their frequencies were significantly different from that of Caucasian, whereas SNP at –863 of the TNF-α gene showed no significant differences between Thai population with

Caucasians and Asian. These data highlight the variability of cytokine gene frequencies in different population groups.

CHAPTER VII

CONCLUSION

The aim of this study was to investigate the polymorphisms of IL-1Ra gene, TNF-α gene, TNF-β gene, IL-4 gene and IFN-γ gene in patients with GD compare with control group and determine the association with GD in Thai population. The results demonstrated that the –863 polymorphisms in the promoter region of the TNF-α gene is the marker for genetic susceptibility to GD in Thai population. The effect of –863A allele was similar to autosomal dominance mode of inheritance. The presence of one A allele (AA or AC) conferred the significant OR of 2 (*P*=0.01, 95% CI=1.16 to 3.44). Although the genetic evidence remains to be further proved, the –863A allele may be involved in susceptibility to GD in part through their higher promoter activity of TNF-α production. TNF-α induces the expression of adhesion molecules, regulatory molecules and HLA class II molecules on thyroid epithelial cells, allowing these cells to present antigens to activated T cells. This presentation of antigen might exacerbate the autoimmune processes involved in the pathogenesis of GD. However, further studies on the functionality of this polymorphism are necessary. Furthermore, as a further step, research in to associations between cytokine genotypes and disease severity, may help both to explain basic biological events and indicate to clinicians ways of predicting, preventing or managing harmful situations in disease with immunological components.

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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.

BIOGRAPHY

Mr. Jeerawat Nakkuntod was born on May 25, 1979 in Nakorn Ratchasima, Thailand. He 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 his master degree.