TNF- ALPHA GENE POLYMORPHISMS IN THAI COPD AND SILICOSIS PATIENTS

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TNF- ALPHA GENE POLYMORPHISMS IN THAI COPD AND SILICOSIS PATIENT.

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) and silicosis are common disease entities in Thailand. Environmental factors especially cigarette smoking and occupational exposures are the greatest contributors to the disease development. Based on the evidence that only a minority of cigarette smokers develops COPD and genetic background may contribute to the disease pathogenesis. This is likely in silicosis that not all individuals develop severe lung fibrosis despite having similar work histories. Although the major determinant of silicosis is the level of exposure to silica containing dust, individual susceptibility to the disease may also play an important role.

Tumor necrosis factor-alpha (TNF-α), a potent proinflammatory cytokine, can damage lung structure and/or sustaining neutrophilic inflammation. It also promotes bronchial smooth muscle proliferation and alters smooth muscle function. The increment of sputum TNF-α levels and TNF-α immunoreactivity in the airways of COPD patients were well recognized. There is also evidence that TNF-α plays an important role in the pathogenesis of silicosis and the experimental inhibition of TNF receptors has been shown to protect mice from the inflammatory and fibrotic effects of silica.

The promoter polymorphism of TNF-α gene was associated with COPD and silicosis in different study populations. The aim of the present study was to evaluate the frequency of TNF- α gene promotor polymorphisms in Thai patients with COPD and silicosis, and compare them with the frequencies in appropriate control subjects; PCR-SSP was used to detect the frequencies of TNF-α. There were no significant differences in the distribution of TNF-α gene promoter among patient with COPD, smoker control subjects, and normal population control subjects. But the distribution of TNF-α -308 allele was significantly different between silicosis patients and population control subjects ($p<0.05$).

KEYWORDS: TNF- alpha GENE / POLYMORPHISM / COPD / SILICOSIS /PCR-SSP
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ความหลากหลายทางพันธุกรรมของยีน TNF-alpha ในคนไทยซึ่งป่วยเป็นโรคปอดอุดกั้นชนิดเรื้อรังและโรคปอดที่เกิดจากการสูดฝุ่นที่มีส่วนผสมของซิลิกา (TNF-ALPHA GENE POLYMORPHISMS IN THAI COPD AND SILICOSIS PATIENT)

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บทคัดย่อ

โรคปอดอุดกั้นเรื้อรัง(COPD) และโรคปอดที่เกิดจากการสูดฝุ่นที่มีส่วนผสมของซิลิกา (silicosis) เป็นโรคที่พบได้บ่อยในประเทศไทย ปัจจัยทางสิ่งแวดล้อมโดยเฉพาะการสูบบุหรี่และการประกอบอาชีพที่ต้องสัมผัสกับฝุ่นซิลิกาเป็นสาเหตุสำคัญที่ทำให้เกิดโรค จากการที่พบว่าในคนที่สูบบุหรี่มีเพียงจำนวนน้อยเท่านั้นที่เป็นโรค COPD ทำให้คาดว่าระบบพันธุกรรมน่าจะเกี่ยวข้องกับการเกิดโรค COPD และเช่นเดียวกับในโรค silicosis พบว่าคนที่มีประวัติการทำงานในลักษณะที่ต้องสัมผัสกับฝุ่นซิลิกาเหมือนกันไม่ได้เป็นโรคนี้ทุกคน ถึงแม้ว่าสาเหตุหลักของการเกิดโรคซิลิโคซิสคือการสูดฝุ่นที่มีส่วนผสมของซิลิกา แต่การตอบสนองต่อการเกิดโรคของแต่ละบุคคลก็น่าจะมีบทบาทสำคัญในการเกิดโรคเช่นกัน

Tumor necrosis factor-alpha (TNF-α) เป็นสารคัดหลั่งที่มีอิทธิพลในการทำให้เกิดการอักเสบต่างๆภายในร่างกาย สามารถทำลายโครงสร้างของปอด และ/หรือกระตุ้นกระบวนการอักเสบ นอกจากนี้ยังทำให้เกิดการแบ่งตัวของของกล้ามเนื้อเรียบของหลอดลมและทำให้การทำงานของกล้ามเนื้อเรียบเปลี่ยนไป ในเสมหะของผู้ป่วย COPD จะมีปริมาณ TNF- α สูงขึ้น ร่วมกับมีกระบวนการทำงานทางระบบภูมิคุ้มกันของ TNF- α ในระบบทางเดินหายใจ ส่วนในโรค Silicosis มีการพิสูจน์แล้วว่า TNF- α มีบทบาทสำคัญในการเกิดโรค โดยการทดลองยับยั้ง TNF receptors ในหนูทดลอง พบว่าสามารถป้องกันการอักเสบและการเกิด fibrotic จากการสัมผัสฝุ่นซิลิกาได้

จากการศึกษาความหลากหลายทางพันธุกรรมของยีนต้นกำเนิด TNF- α พบว่ามีความสัมพันธ์กับการเกิดโรค COPD และ silicosis ในกลุ่มประชากรต่างๆกัน ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาความหลากหลายทางพันธุกรรมของยีน TNF- α ในกลุ่มผู้ป่วย COPD และ silicosis ในประเทศไทย โดยเปรียบเทียบกับกลุ่มควบคุมที่เหมาะสมด้วยเทคนิควิธี PCR-SSP จากการศึกษาพบว่าไม่มีความแตกต่างกันของยีน TNF- α ในกลุ่มผู้ป่วย COPD, กลุ่มควบคุมที่สูบบุหรี่ และกลุ่มประชากรปกติ แต่ในกลุ่มผู้ป่วย silicosis พบว่ายีน TNF-α ในตำแหน่ง -308 มีความแตกต่างอย่างมีนัยสำคัญกับกลุ่มประชากรปกติ

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LIST OF ABBREVIATIONS

Abbreviation	Term
COPD	chronic obstructive pulmonary disease
TNF-α	tumor necrosis factor – alpha
TNF-ß	tumor necrosis factor – beta
A	adenine
G	guanine
C	cytosine
T	thymine
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
HLA	human leukocyte antigen
SNPs	single nucleotide polymorphisms
TNFR	tumor necrosis factor receptor
LT- alpha	lymphotoxin alpha
FasL	Fas ligand
RANKL	receptor activator of TNF-kappaB ligand
CD40L	CD40 ligand
TRAIL	TNF- related apoptosis- inducing ligand
TRADD	TNFR1-associated death domain protein
FADD	Fas asssociated death domain protein

LIST OF ABBREVIATIONS (continue)

Abbreviation	Term
RIP	receptor- interacting protein
RA	rheumatoid arthritis
SSCP	single strand conformation polymorphisms
UTR	untranslated region
PCR-SSOP	polymerase chain reaction – sequence specific oligonucleotide probe
PCR-RFLP	polymerase chain reaction- restriction fragment length polymorphism
PCR-SSP	polymerase chain reaction – Sequence specific primers
SLE	systemic lupus erythematosus
SE	shared epitope
IDDM	insulin dependent diabetic mellitus
BMI	body mass index
MS	multiple sclerosis
CPE	chronic pulmonary emphysema
ICU	intensive care unit
FEV1	forced expiratory volume in 1 second
CWP	coal workers' pneumoconiosis
ILO	international labour office
dNTP	deoxynucleotide triphosphate
FVC	force vital capacity
bp	base pair
UCLA	University of California at Los Angeles

LIST OF ABBREVIATIONS (continue)

Abbreviation	Term
RBC	red blood cell
µl	microliter
ml	milliliter
SDS	sodium dodicyl sulphate
Guanidine HCl	guanidine Hydrochloride
M	molar
g	gram
ddH_2O	deionizer distill water
TE	tris-EDTA
EDTA	ethylenediamine tetra acetic acid
EtOH	ethanol
µM	micromolar
mM	millimolar
TBE	tris-boric EDTA
nm	nanometer
mv	millivolt
uv	ultra violet
CFTR	cystic fibrosis transmembrane regulator
HIV	human immunodeficiency virus

CHAPTER I

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) and silicosis are common disease entities in Thailand. Cigarette smoking and occupational exposures are firmly established as the most important risk factor for the disease development. Only a minority or about 10% to 15% of smokers develop clinically significant COPD. There is likely in silicosis, not all individuals develop severe lung fibrosis despite similar work histories. This strongly suggests that genetic factors may influence the extent of these diseases.

Tumor necrosis factor-alpha (TNF-α) is a multifunctional cytokine. TNF-α could promote tracheal smooth muscle proliferation (1) and alter smooth muscle function (2), cell differentiation, apoptosis, modulation of immune responses and induction of inflammation. Overproduction of TNF-α as a result of age or genetic defects has been implicated in many inflammatory diseases. The increment of sputum TNF-α levels and TNF-α immunoreactivity in the airways of COPD patients were well recognized (3, 4). There is also evidence that TNF-α plays an important role in the pathogenesis of silicosis, and the experimental inhibition of TNF receptors has been shown to protect from the inflammatory and fibrotic effects of silica in mice.

The polymorphism TNF-α gene promoter has been associated with higher baseline and induced expression of TNF-α. A polymorphism consists of a guanine to adenine substitution. A polymorphism at position -308 of the TNF-α gene promoter is associated with alteration of TNF-α secretion in vitro (5), has heterogenous results concerning the association with COPD and silicosis in different study populations.

Previous studies, in Taiwanese populations, TNF-α polymorphisms confers a higher risk to the development of chronic bronchitis (6). In Japanese populations,

TNF-α -308 1/2 alleles were significantly associated with the presence of smoking related COPD (7). However, there were no significant differences in TNF1/2 allele frequencies in Caucasian (8). In Black South African miners demonstrated the significant associations between the -238, -376 and -308 A alleles of the TNF-promoter only in severe patients of silicosis according to the radiographic findings(9).

Whether this polymorphism contributed to the genetic susceptibility of COPD and silicosis in Thailand is the aim of this study. The PCR-SSP was used to detect the frequencies of TNF-α polymorphism. The significant differences in TNF-α gene polymorphism between patients and controls were compaired by X^2- test.

CHAPER II

OBJECTIVES

The aim of the present study was to evaluate the frequencies of TNF-α gene promotor polymorphisms in Thai patients with COPD and silicosis, and compare them with the frequencies in appropriate control subjects.

Usage expected from this research

1. To clarify the correlation of TNF gene in COPD.
2. To clarify the correlation of TNF gene in silicosis.

Hypothesis

TNF-α gene promoter polymorphism were associated with pathogenic in Thai COPD and silicosis patients.

CHAPTER III

LITERATURE REVIEW

Approaches to genetic polymorphism analysis

Genetic polymorphisms are defined as variations in DNA that are observed in 1% or more of the population. Genetic polymorphisms may alter protein structure and function through a single nucleotide base substitution in a gene's coding region, and may increase or decrease gene expression either by affecting mRNA stability when occurring in a gene's 3′ untranslated region or by altering transcription factor binding when occurring in the 5′ promoter region. Alternatively, a polymorphism may have no discernable effect on the protein products and may lie within DNA regions that are not involved in gene transcription or translation. Polymorphisms that exist in these regions as variations in repeat sequences throughout the genome have served the basis for genetic linkage analysis (10).

The study of genetic polymorphisms promises to help define pathophysiologic mechanisms, to identify individuals at risk for disease and to suggest novel targets for drug treatment. The methodology to study polymorphisms is simple, requiring only access to a polymerase chain reaction machine, funding for reagents, and DNA samples from cases and controls. The seemingly unlimited potential of genetics to help predict who will get lung disease or who, once diagnosed with disease, will have an unfavorable prognosis has inspired many investigators to jump on the bandwagon of studying genetic polymorphisms. While progress in understanding and treating pulmonary diseases has occurred through investigating genetic polymorphisms, the limitations and potential pitfalls of this approach may be under-appreciated.

Association studies are limited to evaluating DNA polymorphisms near or within candidate genes. To perform a genome screen to search for candidate genes, linkage analysis using families or affected siblings is required. Linkage analysis is

comprehensive and locates genes that exert a major effect on disease susceptibility, but linkage analysis has relatively low power and will fail to detect genes conferring only mild to moderate disease risk. For instance, if a disease susceptibility allele exerts a twofold disease risk compared with the wild-type allele, several hundred to several thousand families need to be typed, a sample size that may not be achievable. Association studies have greater power, but associations are detected over much smaller genetic regions (thousands of base pairs) compared with that detected by linkage analysis (millions of base pairs). To perform a genome scan with association studies, tens of thousands of markers would be needed, which is not possible with current technology; although it is anticipated that this may soon be possible (11).

TNF alpha

Tumor necrosis factor (TNF) gene is located in the major histocompatibility complex (MHC) class III region on chromosome 6 p21. In total, 7 polymorphic sites have been described in and around TNF. The polymorphisms include five microsatellites (a-e), ten biallelic polymorphisms and a C insertion site. In TNF, 7 polymorphisms have been described; -308, -224, -238, +70 C ins, +488, -163 and –376. The transcribed exons are boxed and the untranslated regions of the exons are shared.Nucleotide positions of the polymorphisms were taken from the literature and calculated from the GenBank sequence Z15026. The numbers circled correspond to the reaction numbers outline in Table1. Four TNF haplotypes were observed in the 201 United Kingdom controls and were assigned allele numbers TNF 1-4 according to frequencies in the control population (Figure 1) (12).

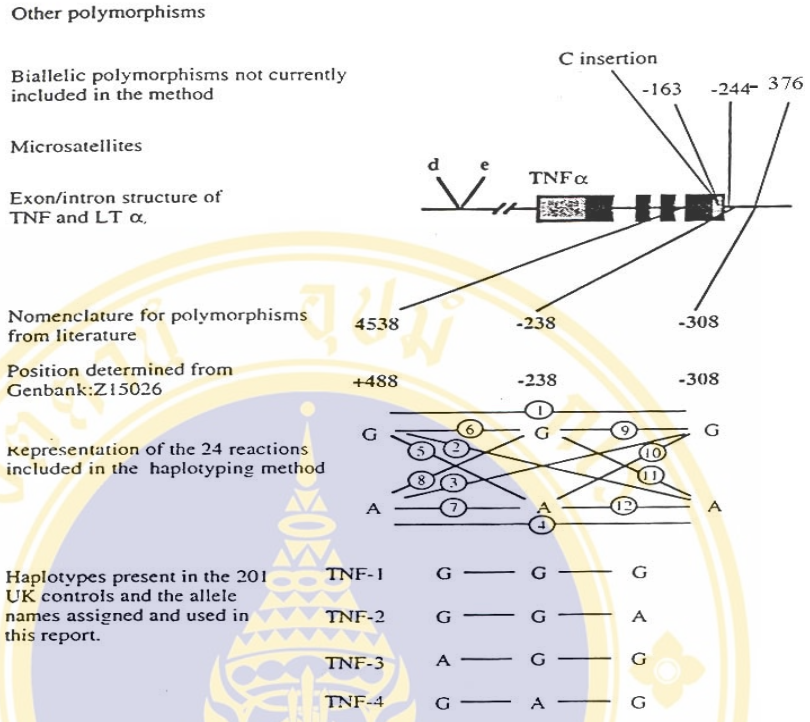

Figure1 Diagrammatic representation of the TNF polymorphisms

TNF family members play important roles in various physiological and pathological processes, including cell proliferation, differentiation, apoptosis and modulation of immune responses and induction of inflammation. TNF acts through two receptors, TNFR1&2. TNFR1 is expressed by all human tissues and is the major signaling receptor for TNF-alpha. TNFR2 is mostly expressed in immune cells and mediates limited biological responses. TNFR2 binds both to TNF-alpha and TNF-beta. TNF-alpha is representative of a still growing family of trimeric cytokines and cell-surface proteins, such as lymphotoxin-alpha (LT-alpha), Fas ligand (FasL), receptor-activator of TNF-kappaB ligand (RANKL), CD40 ligand (CD40L), and TNF-related apoptosis-inducing ligand (TRAIL). Exposure of cells to TNF-alpha can result in activation of a caspase cascade leading to apoptosis. However, more commonly, the binding of TNF-alpha to its receptors causes activation of two major transcription factors, AP-1 and NF-kappaB, which in turn induces genes involved in chronic and acute inflammatory responses (13). The binding of TNF-alpha to TNFR1 activates

TNFR1-associated death domain protein (TRADD), which interacts with Fas-associated death domain protein (FADD) to induce apoptosis (14). The binding of TNF-alpha to TNFR1 can also produce an opposite effect if TRADD interacts with TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP) to activate NF-kappaB, resulting in gene expression and cell survival. TNFR2 triggering can lead to NF-kappaB activation, but does not result in cell death. Importantly, the suppression of apoptosis, which is mostly dependent on NF-kappaB, augments the inflammatory response to TNF-alpha. Overproduction of TNF as a result of age or genetic defects has been implicated in many inflammatory diseases, such as rheumatoid arthritis, which results from TNF induced apoptosis of cartilage cells. TNF pathway is illustrated in Figure 2.

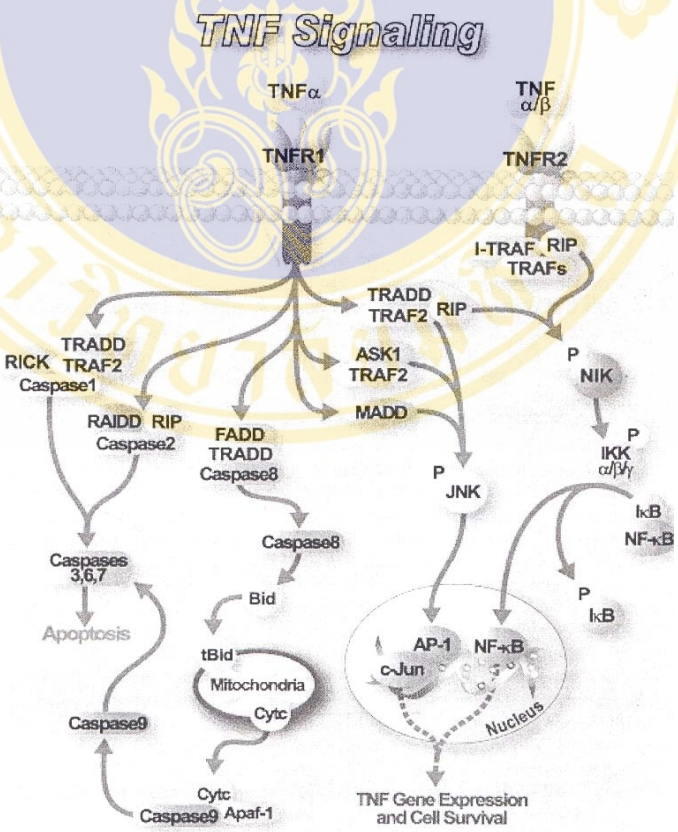

Figure 2 TNF Pathway

TNF single nucleotide polymorphisms (SNPs)

There are many SNPs within the TNF-α gene. In the promoter, these are at positions (relative to the transcription start site) -1031 (T→C), -863 (C→A), -857 (C →A), -851 (C→T), -419 (G→C), -376 (G→A), -308 (G→A), -238 (G→A), -162 (G →A), and -49 (G→A), although those at positions -419, -163, -49 are rare in Caucasians (15). Hence, the 5′ region of the TNF-α gene is highly polymorphic. In addition, there is an insertion of a cytosine at position +70 in the first exon, a G→A substitution at position +488 in the first intron (16), and a deletion of a guanine at position +691 in the first intron of the TNF-α gene (17).

By contrast, the 3′ region of the TNF-α gene appears to be highly conserved. Waldron et al studied the TNF 3′ UTR in rheumatoid arthritis (RA) patients and control. They analyzed >800 bp of the 3′ UTR of the TNFα gene using SSCP and sequencing in a total of 38 subjects with or without RA. No polymorphisms were detected (18), which is in agreement with the study of Becker, conducted in patients with connective tissue disease or type I diabetes. However, he has recently found a rare SNP in the 3′ UTR of TNF-α of Caucasian subjects, a T→C substitution situated 322 bases downstream the last exon. This polymorphism was not associated with RA (19).

TNF SNPs and TNF-α production

There are many SNPs within the TNF-α gene promoter. The effect of SNPs on transcription factors binding has not been fully explored. Many studies have concentrated on one SNP in isolation of others. The -308 G/A SNP have been the most studied polymorphism.

In vitro stimulation of TNF-α production by cells obtained from -308*G/G homozygous individuals and G/A heterozygote individuals exhibited conflicting results. Two studies have reported higher TNF-α production by cells from G/A donors than by G/G group (20, 21). Four other studies have reported no significant effect (22, 23, 24, 25). It is interesting to note that the above six studies used different LPS concentrations, 1 ng–10 μg/ml. In addition, the number of the G/A genotype group

was in quite small, approximately 6–16 individuals, resulting in unability to detect any significant difference between the genotypes.

Gene reporter assays have been employed to investigate the -308 SNP. Again, different results were reported. Three studies suggested that the A allele does influence TNF-α gene transcription (5, 26, 27), while three other have drawn a contradictory conclusion (28, 29, 30). 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 final conclusion.

Similar conflicting results have been demonstrated for the -238 SNP. One study showed that the -238*G allele was associated with high TNF-α production (24), but this was not confirmed in another study (22)

Circulating TNF-α level does not seem to correspond with the -238 and -308 TNF promoter polymorphisms (24, 31, 32). However, circulating TNF-α levels might be under a multifactorial regulatory process. Local TNF-α concentration might be of greater importance and under more control by specific polymorphisms.

Another polymorphism at position -376 in the TNF-α gene promoter was reported to be differentially regulated by Oct-1, such that the A allele binds the Oct-1 proteins while the G allele do not (33).

In fact, linkage disequilibrium is strong in this area and it may be difficult to study the role of an SNP in isolation. In some populations the -376*A allele is in allelic association with the -308*G and -238*A alleles (33). While this seems true in Caucasians, Africans from the Gambia did not show this allelic association. They have also observed a strong allelic association between -238*G, -308*A, and -376*G, which was found to encode high TNF-α production in vitro.

Functional studies of the TNF polymorphism at position -863 (C→A) revealed that this site binds NFκB, both p65-p50 and p50-p50 dimers. The base substitution at

this position inhibits p50-p50 binding, and this may reduce the enhancing effect of NFκB in TNF-α activation (34).

Polymorphisms within the 3′ UTR may be of importance in TNF-α regulation. Deletion of the 3′ UTR of TNF-α in the mouse led to abnormally stable mRNA, and TNF-α was produced by cells that normally do not synthesize TNF (35). The polymorphism that they have identified in the 3′ UTR of the human TNF-α gene is rare and functional studies are underway to evaluate its influence on TNF-α production.

Associations between TNFα polymorphisms and disease

Over-expression of the TNF-α gene in mice leads to arthritis (36). Conversely, when mice homozygous for the TNF-α gene knockout were infected with *Corynebacterium parvum*, there was little or no initial response, but the mice went on to develop a severe and fatal inflammatory reaction (37). By contrast, normal mice developed an early inflammatory response that resolved. This suggests that TNF-α has a dual function, being proinflammatory in the initial infection and then antiinflammatory or immunoregulatory in the later phases of the response (37).

These observations suggest that polymorphism in the human TNF-α gene encoding high TNF-α levels may be important in the susceptibility or severity of arthritic diseases and in other inflammatory conditions.

Association between TNF polymorphisms and infection

In multiply injured patients with severe sepsis the TNF-ß NcoI polymorphism (TNFB2 allele) acts as a predictor of severe post traumatic sepsis and increased levels of circulating TNF-α (29, 38). On the other hand, the -308 TNF-α polymorphism was not associated with survival in severe sepsis (29). How can this result be interpreted? The authors measured circulating levels of soluble TNF-α, but did not attempt to measure TNF-ß. Whether the assay was specific to TNF-α or can detect TNF-ß is not clear. The NcoI polymorphism within the first intron of TNF-ß is known to be associated with high and low TNF-α production, and is in linkage disequilibrium with an amino acid change at position 26 in the coding region of TNF-ß (39). Therefore, it

is unclear whether this effect in sepsis is solely due to the genetic regulation of TNF-α or not. Sepsis can be induced by both gram negative and gram positive bacteria. While the gram negative bacteria produce LPS, a strong inducer of TNF-α production by macrophages, gram positive bacteria such as *Staphylococcus* can produce superantigens that are potent nonspecific stimulators of T cells. One other factor that can add to this puzzle is linkage disequilibrium with other genes within the MHC region, including the HLA DRB1 locus and the complement genes. The HLA DR molecule may influence the presentation of antigens to T cells while complement may play a role in the clearance of infectious agents.

It is interesting to note that the -308*A allele has been associated with severe septic shock and death (15). Why should different polymorphisms within the TNF locus be associated with these two conditions, septic shock and sepsis? Many factors may contribute to these findings, possibly including the distribution of HLA DRB1 haplotypes within the population of study. In Mira's study the patients were collected from seven French ICU centres while in Stuber's studies all patients were of German origin. Other factors might include the type of surgery or trauma and the bacterial type.

In a pilot study of neonates with bacterial sepsis, Weitkamp et al. failed to demonstrate an association between TNF-ß NcoI polymorphism similar to that in adults with post-surgical sepsis (40).

In parasitic infections, the -308*A allele has been associated with a fourfold increase in risk for cerebral malaria and a sevenfold increase in risk for development of serious neurological consequences (41). Similar but independent risk for the development of cerebral malaria was also demonstrated for the -376*G allele in malaria-infected individuals from the Gambia and Kenya (32).

Several studies have investigated the association between TNF alleles and viral infections. The -238*A allele was associated with chronic infection with hepatitis B virus. No similar findings were found for -308 polymorphisms (42). Similar findings were also reported for hepatitis C virus infection, the -238*A allele being associated with chronic hepatitis while the -308 alleles were not (43). This was not due to linkage

disequilibrium with either DRB1 or HLA B. In a recent study, Yee et al. found that both -238*A and -308*A were significantly associated with liver cirrhosis in hepatitis C (44). In HIV, they have shown that the TNFc2 allele was associated with slow progression of the disease (45). This result was not confirmed with any of the SNPs in the TNF-α gene including -163, -238, -308, and -376 (46). Results from HLA studies in HIV have suggested that the HLA DR3, B8, A1 haplotype is associated with faster progression. This haplotype is in linkage disequilibrium with the TNFc1 allele (47), and is associated with higher TNF-α production. Thus the TNF c alleles may relate to the outcome of viral infection.

TNF polymorphisms and autoimmune diseases

In autoimmune diseases, TNF-α is one of the most studied genes. As most autoimmune diseases are associated with HLA, results of TNF polymorphism studies could be interpreted by linkage disequilibrium with HLA or, indeed, vice versa.

SLE is associated with the HLA DRB1*0301, B8, A1 haplotype. In UK Caucasians, found that the TNFa2, b1, and d3 alleles are associated with SLE. This may be explained by the strong linkage disequilibrium between these alleles and the HLA DR3, B8, A1 haplotype (48). In Greek SLE patients, in whom HLA DR2 haplotypes are associated with SLE, different TNF microsatellite alleles, especially TNFa11, were increased in frequency. These were in linkage disequilibrium with HLA DRB1*15 haplotypes (49).

The -308*A allele was originally shown to be associated with SLE in Caucasians. This allele forms part of the SLE haplotype, HLA DR3, B8, A1 (50). More recently, Rood et al. found that the -308*A allele association with SLE is independent of HLA DRB1*0301. No association with the -238 alleles was found (51).

In RA, TNF-α is the driving force of the inflammatory response of the joints (52). While TNF-308 alleles were not found to be associated with RA (53), the TNF microsatellite alleles were. They were the first to show the association of the TNF a6 allele with RA. TNFa6, b5, c1, and d4 were found to form part of the HLA DRB1*0401, B44 haplotype (47). Other microsatellite alleles were found also in RA

patients, a2, b1, c2, d5 that form part of the HLA DRB1*0401, B62 haplotype. This haplotype was more frequent in male RA patients while the HLA DRB1*0401, B44 was increased in female patients (48).

In a study of RA patients from Northwestern Spain, they found by logistic regression analysis, that the TNFc1 allele was independent of HLA DR4 or HLA-DRB1 shared epitope (SE) status (54). TNFc1 was not associated with severe RA, but TNFa2 was associated with erosive disease and was independent of HLA DR4 and SE. As discussed earlier, unless it is part of the HLA DR3, A1, B8 haplotype, TNFc1 is associated with low TNF-α production, but still carries a risk for RA susceptibility. This could be explained by the presence of another susceptibility gene in close proximity to TNF-α and in linkage disequilibrium with the TNFc alleles. The TNFa2 allele has been reported to be associated with high TNF-α production in vitro. This may explain its association with severity in RA. A recent study of familial RA in Spanish patients showed that the TNFa6, b5 haplotype was preferentially transmitted to affected offspring independent of HLA DR and the SE (55). Overall, these results suggest that the TNF region encompasses a susceptibility locus to RA that is independent of HLA DR.

A recent study in ankylosing spondylitis Rudwaleit et al. found that HLA-B27 positive patients had lower numbers of TNF-α positive T cells compared with controls. They also found in B27 positive patients heterozygosity for the -308 polymorphism was associated with a higher percentage of TNF-α positive T cells than homozygous TNF - 308*G/G (56).

IDDM is strongly associated with inheritance of the HLA DR3 and DR4 antigens. The TNF microsatellite a2 alleles and the TNFB*2 allele (TNF-ß NcoI) were significantly increased in IDDM patients. These alleles are associated with high TNF-α production in vitro (57). Monos et al found that TNFa1, b5 haplotypes are increased in frequency in IDDM and are in linkage disequilibrium with the HLA DRB1*0301, B18 haplotype (58).

The -308*A/A homozygous genotype was recently shown to be associated with excessive fat accumulation in females [body mass index (BMI)], but not in men (59).

An earlier study had suggested an association between the -308 polymorphism and lower BMI, although in that study cases were not stratified according to gender (60).

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system associated with HLA DR2 in North Europeans and North American Caucasians. The TNFc1, a11, b4 microsatellite alleles have been associated with MS, possibly because of their association with the HLA DRB1*1501 haplotypes (61). The TNF -308 polymorphism was investigated by several groups (23, 24, 62, 63, 64). None of these studies showed any significant association with MS. By contrast, Huizinga et al. found that the TNF-238 alleles were associated with severe MS (24). As with many other diseases associated with HLA/TNF region, it is very difficult to dissect out the gene(s) contributing to MS susceptibility within the MHC region. They believe a haplotype analysis of a large sample size containing multiple ethnic groups could resolve this issue.

TNF polymorphisms and transplantation

Biopsies from rejecting kidney or heart transplants show the presence of an inflammatory infiltrate of cells capable of producing TNF-α. Studies in heart transplant recipients (65), kidney (66, 67), and liver (68) transplant recipients revealed an association between TNF-α -308 polymorphisms and acute rejection. Death of heart transplant recipients as a consequence of irreversible acute rejection of their graft is exclusively confined to those of the TNF-α -308*A genotype (31, 69).

Poli et al. found that the incidence of acute rejection episodes, vascular damage, and creatinine levels at one month post kidney transplantation were significantly associated with the -308*A allele. Logistic regression analysis demonstrated that the TNF -308*A and donor age were significantly and independently associated with acute renal graft failure (70).

Cavet et al. found that mortality of bone marrow transplant recipients is higher in those who are homozygous for the TNFd3 microsatellite allele compared with those who lack this allele (71). On the other hand, Turner et al. found that the TNFd3 allele goes with higher TNF-α production in vitro, but was not associated with acute heart rejection (65). Sahoo et al. investigated the role of the NcoI polymorphism in renal

transplantation (72). Their results did not support a function for this polymorphism in rejection, but was associated with susceptibility to infection in transplanted patients. Asano et al. reported an association between the TNFa9 microsatellite allele and rejection of renal transplantation in Japanese patients (73). This allele was in linkage disequilibrium with HLA B35. However, the TNFa9 allele was more closely related to rejection than HLA B35.

TNF polymorphisms and Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a term that refers to a variety of chronic lung disorders, which include chronic pulmonary emphysema (CPE) and chronic bronchitis (74). In older textbooks and medical literature COPD included asthma and other forms of obstructive lung disease but this is not true today. COPD is characterized by the progressive deterioration of airflow limitation that is not fully reversible. The lack of reversibility is the major criterion that is used to distinguish COPD from asthma. COPD is basically a benign disease, but the prognosis is so poor thinks to its mortality rate similar to that of some malignant disease. With COPD it is difficult to breathe because airflow in and out of the lungs is restricted. Air that normally flows easily into and out of the lungs is slowed down by mucus lying in the breathing tubes and swelling, and the breathing muscles contract more than they should (75). The lungs aren't working as well as they could and they will become worse over time. A mixture of chronic cough, increased sputum and shortness of breath characterize COPD.

COPD is currently the fourth leading cause of death in the United States. It has increased in incidence 41.5% since 1982. Sixteen million Americans with COPD have been identified, and it is estimated that 30-35 million Americans may be afflicted. It is the only major cause of death for which the numbers are rising. While overall mortality decreased 22%, mortality from heart disease (the number one cause of death) and cardiovascular disease decreased 45% and 58% respectively, mortality from COPD increased 32.9% from 1979 to 1991. Costs of COPD patients are 2.4 times the cost for all Medicare beneficiaries and are at an estimated $40 billion annually. Many feel that unless current trend is reversed, COPD is unfortunately likely to be the disease of the new millennium (75).

In Canada, an estimated 750,000 people have a form of COPD. However, COPD remains a very under diagnosed disease and estimates suggest that many more people have COPD but have not yet been diagnosed (76). Nevertheless, only 10-15% of smoker develops the severe impairment of pulmonary function associated with COPD (77). Moreover, the factors that determine the susceptibility to cigarette smoking and disease progression are poorly understood. Among those factors, the genetic contribution to the disease has been implicated (78). Although the nature of the genetic influences remains undefined, factors that regulate the inflammatory responses to direct exposure to inhaled insults are important in the pathogenesis of COPD (79).

In the United Kingdom, respiratory conditions are the third commonest cause of chronic sickness in working age 45-64 years, is the commonest cause of respiratory related death, and COPD accounts for 56% of days of certified incapacity due to respiratory conditions in males. In 1989, an estimated $7 billion was spent on provision of care for patients with COPD in the USA, with a further cost of $8 billion due to lost productivity (80).

In Thailand, the studies about The Cost of Treatment for COPD Patients at Ramathibodi Hospital in the period July 1, 2000 - June 30, 2001 consisted of thirty COPD patients (11 mild cases, 13 moderate cases, and 6 severe). The cost of treating the COPD patients with mild, moderate and severe disease, taking the average of medical costs per person per visit, was 850, 1,100 and 1,041.50 baht, respectively. Medical costs for inpatients per person per visit were 2,200 and 2,100 baht according to the severity of disease. Patients admitted to the Intensive Care Unit (ICU) incurred a cost per person per day for moderate and severe case of 4,775 and 5,888.42 baht respectively. Conclusion, an important factor affecting the medical cost of treating COPD patients was severity of disease.

More than two thirds of Thai smokes ignore early warning sign of respiratory ailments, according to a study of smoking habits in six Asian countries. Although 69% of interviewees reported experiencing one or a combination of COPD symptoms, a majority felt the symptoms were not serious enough to consult a doctor. Only 26% of them went to see doctors, while 23% chose to ignore the symptoms, 25% chose to drink lots of water, and 42% opted for self-medication or traditional herbs. From

collaboration study, up to 90% of COPD patients are smokers or ex-smokers and 7.1% of people over 60 suffer from COPD. Typically, a COPD patient has smoked one pack a day for over 20 years. However, it does not mean that those who smoke less will not get the disease because people have different levels of genetic susceptibility to COPD. (81)

That only 10–20% of cigarette smokers develop symptomatic COPD suggests that genetic factors are likely to be important. In addition, several studies have shown an increased prevalence of COPD within families. COPD thus appears to be ripe to investigate genetic polymorphisms in disease susceptibility (82). The genes which have been implicated in the pathogenesis of COPD are involved in antiproteolysis, metabolism of toxic substances in cigarette smoke, airway hyperresponsiveness and the inflammatory response to cigarette smoke (83). The genes involved in the pathogenesis of COPD are summarized in figure 3.

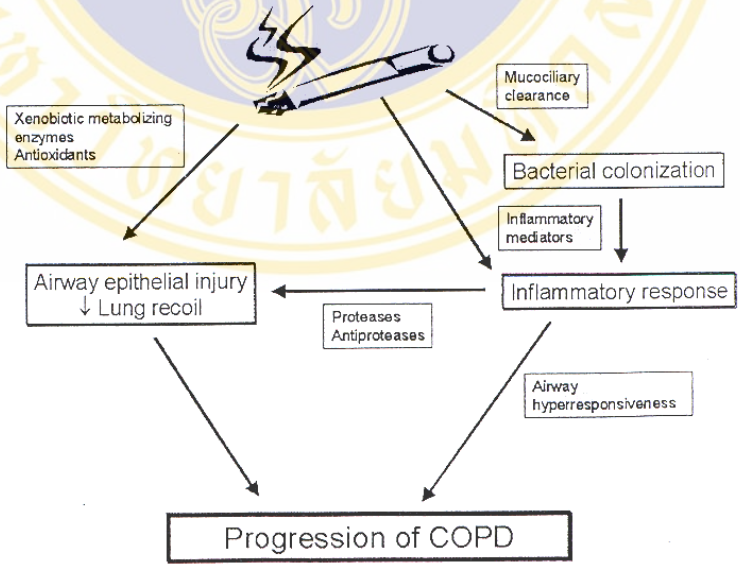

Figure3 Summary of pathway and possible candidate genes involved in the pathogenesis of COPD

Huang et al. investigated the distribution of TNF-α polymorphism in Taiwanese populations with a case-control study. The genotype was determined in 42 male patients with chronic bronchitis, 42 sex-, age-, and smoking index-matched control subjects, and 99 random-sampled schoolchildren. The authors reported here that the TNF2 allele is overrepresented in the patient group. The allele frequency of TNF2 is 5.1% in the schoolchildren, 2.4% in the control group, and 19% in the bronchitis group ($p < 0.01$). Carriage of the TNF2 allele confers a higher risk to the development of chronic bronchitis (odds ratio = 11.1, 95% CI = 2.89-42.57). The results demonstrate the important pathologic role of TNF-α in chronic bronchitis and suggest that greater inflammatory response may predispose an individual to this disease (6).

Sakao et al. have argued that TNF-α may be involved in the development of COPD. TNF-α has been reported to be elevated in bronchoalveolar lavage, bronchial biopsies and induced sputum of COPD patients. They compared TNF-α -308 1/2 allele frequencies in 106 Japanese patients with 110 asymptomatic smoker/ ex-smoker control subjects matched for sex and age, and 129 population control blood donors. The authors reported that TNF-α-308 1/2 alleles were significantly associated with the presence of smoking-related COPD. Allele frequencies were significantly different among the groups: in patients with COPD, the 1/2 allele frequencies were 0.835/0.165; in smoker/ex-smoker control subjects, 0.918/0.082; and in the population control subjects, 0.922/0.078. However, the TNF-α -308*2 allele was not found to be associated with COPD in a white population (7). Furthermore, studies have been inconsistent in demonstrating an association with the presence of the TNF-α -308*2 allele in a number of inflammatory diseases such as sarcoidosis and asthma (84, 85, 86).

Higham et al. performed a study investigating association of the polymorphism with smoking-related COPD in Caucasians. Frequencies of TNF1/2 alleles in 86 Caucasians (52 males) with COPD were compared with 63 (52 males) asymptomatic smoker/exsmoker control subjects and a population control of 199 (99 males) blood donors. Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism technique on genomic deoxyribonucleic acid (DNA) obtained from peripheral blood. There were no significant differences in TNF1/2 allele frequencies between groups: 0.85/0.15 in COPD, 0.85/0.15 in smoker control subjects,

0.83/0.17 in population control subjects. Within the COPD group there was no association of TNF1/2 alleles with indices of airflow obstruction (% predicted forced expiratory volume in one second (FEV1) and % predicted FEV1/vital capacity ratio) nor gas transfer (% predicted carbon monoxide transfer coefficient and % predicted carbon monoxide diffusing capacity of the lung). It is concluded that: 1) the tumour necrosis factor gene promoter allele does not influence the risk of developing chronic obstructive pulmonary disease in a Caucasian population of smokers; and 2) there is no association of the tumour necrosis factor gene promoter genotype with severity of neither airflow obstruction nor degree of emphysema in chronic obstructive pulmonary disease (8).

Pattzzo et al. study the frequencies of the two TNF gene complex polymorphisms in patients with COPD and bronchiectasis. They studied the frequencies of these polymorphisms in 66 subjects with COPD and in 23 subjects with disseminated bronchiectasis and compared them to the frequencies in 98 healthy control subjects and 45 subjects with nonobstructive pulmonary disease. Genomic DNA samples were extracted, and TNF-α and Ltα NcoI polymorphisms were detected after polymerase chain reaction by restriction digestion. The TNF-308*2 allele was detected in 11% of COPD individuals, 15% of bronchiectasis patients, 10% of healthy control subjects, and 18% of subjects with nonobstructive pulmonary disease. The LtαNcoI*1 allele was detected in 28% of COPD individuals, 30% of bronchiectasis patients, 29% of healthy control subjects, and 29% of subjects with nonobstructive pulmonary disease. We found evidence of linkage disequilibrium between the two loci (Δ= 0.068). They conclude that the TNF gene complex, at least in Caucasoid individuals and for the considered polymorphisms, does not seem to play a major role as genetic risk factor in COPD and bronchiectasis (87).

TNF polymorphisms and Silicosis

Silicosis is the chronic fibrosis disease of the lungs produced by the prolonged and extensive exposure to free crystalline silica. When workers inhale crystalline silica (dust), the lung tissue is manifested by persistent overproduction of inflammatory mediators, leading to alveolar damage, fibroblast proliferation, collagen deposition, and granuloma formation. This fibrotic condition of the lung is called silicosis. If the

nodules grow too large, breathing becomes difficult and death may result. Silicosis victims are also at high risk of developing active tuberculosis. The disease is prevalent among workers in the coal mining, manufacturing, and construction industries and contributed to 4882 deaths between 1979 and 1992 (88).

In Thailand, the most common and serious sorts of pneumoconiosis are silicosis (resulting from silica dust). According to the silicosis monitoring program carried out by Division of Occupational Health in cooperation with Provincial Health Offices, there are about 6,712 workplaces that have risk to silicosis, and about 181,907 persons are at risk. The industries that have risk to silicosis are quarrying, sand blasting, glass industries, etc. Division of Epidemiology, Office of the Permanent Secretary, Ministry of Public Health reported 24 silicosis cases in 1993, which is 8 times higher than cases detected in 1992 (3 cases). Those cases were found mostly in Loei (15 cases) and Lopburi provinces (7 cases). However, in 1994, reported silicosis cases decreased to 10.

Animal models have suggested that TNF-α play significant roles in silica- induced lung damage (89, 90, 91). Experimental animal and clinical studies indicate that tumor necrosis factor-alpha (TNF-α) derived primarily from alveolar macrophages and type II epithelial cells in the lung are important in regulating these mediators in silicosis. In this respect, increased expression of inflammatory cytokines corresponds to pathological change in lungs of silicotic rodents (89, 90, 91, 92). A direct role has been demonstrated in TNF-α deficient mice, which are resistant to developing fibrosis from silica (93, 94). Furthermore, the expression of the TNF-α transgene in the murine lung causes spontaneous alveolitis, alveolar disruption, and a progressive fibrotic reaction (95). In humans, TNF-α is released from peripheral blood monocytes in coal miners with coal workers' pneumoconiosis (CWP), while continuous release of TNF-α from alveolar macrophages occurs in miners with progressive massive fibrosis and with simple pneumoconiosis (96, 97). The local release of TNF-α from humans has also been shown to coincide with pathogenesis of the disease (98, 99).

It has been suggested that a number of genetic and epigenetic factors modify the severity of chronic inflammatory diseases. Susceptibility genes, including single nucleotide polymorphisms (SNPs), which quantitatively affect the production of

inflammatory mediators fall into this category. Because not all individuals develop severe lung fibrosis despite similar work histories, it is assumed that genetic factors influence the extent of disease (moderate or severe). In view of this assumption and the paucity of information on the direct role of TNF-α on progressive silicosis in humans, they investigated with these genes and the clinical severity of the disease. Specifically, the frequency of the TNF-α -308 variants in relationship to silicosis severity was examined.

Yucesoy et al. Examined TNF-α frequency in 325 ex-miners with moderate and severe silicosis and 164 miners with no lung disease. The odd ratio of disease for carriers of the minor variant, TNF-α -238 was markedly higher for severe silicosis (4.0) and significantly lower for moderate silicosis (0.52) (Table1). Regardless of disease severity, the odd ratios of desease for carries of the TNF-α -308 variants were elevated. TNF-α -308 also showed a strong relationship with disease, as the presence of the variant was associated with an increase odd for both moderate and severe disease (adjusted odd ratios of 3.6 and 1.6, respectively) (100).

Corbett et al. Examined Polymorphisms at positions -308, -238, and -376 in the TNF-alpha promoter region were compared in nine patients with severe silicosis with International Labour Office (ILO) grade 3 modularity, 112 patients with less severe silicosis (ILO grades 1/1 to 2/2), and 120 black South African gold miners without silicosis (ILO grades 0/0) in an age-frequency-matched case-control study. There were no significant differences between miners with less severe silicosis and controls at any loci in the TNF-alpha promoter region, but miners with severe silicosis were significantly more likely than controls to have -238A (33% versus 6%, Fisher's exact p value = 0.022) and -376A (33% versus 5%, Fisher's exact p value = 0.016). These alleles were in linkage disequilibrium ($p < 0.001$), and so were not independent. The association remained significant (Fisher's exact p values = 0.011 and 0.011, respectively) when analysis was limited to the majority tribe, which included all subjects with severe silicosis. Subjects with severe silicosis were also significantly more likely to have the -308A allele (Fisher's exact p value = 0.034), but this result was confounded by ethnicity and was not significant within Basotho tribe members (Fisher's exact p value = 0.15). TNF-alpha promoter polymorphisms are associated with severe,

but not less severe, silicosis in this population. A predominant effect on disease severity, rather than on disease frequency, appears to be a general feature of promoter polymorphism in diseases in which TNF-alpha has a critical role (9).

Table 1 Summary of TNF-α gene polymorphisms from review literature

Scientist	Study	result
Huang et al.	TNF308*2 in Taiwanese chronic bronchitis patients by PCR-RFLP	TNF2 allele confers a higher risk to the development of chronic bronchitis (odds ratio = 11.1)
Sakao et al.	TNF-∝-3081/2 in Japanese COPD patients by PCR-RFLP	TNF-∝-308 1/2 alleles were significantly associated with the presence of smoking-related COPD - in patients with COPD, the 1/2 allele frequencies were 0.835/0.165 - in smoker/ex-smoker control subjects, 0.918/0.082 - in the population control subjects, 0.922/0.078
Higham et al.	TNF1/2 in Caucasians COPD patients by PCR-RFLP	no significant differences in TNF1/2 allele frequencies - 0.85/0.15 in COPD, 0.85/0.15 in smoker control subjects - 0.83/0.17 in population control subjects

Scientist	Study	Result
Patuzzo et al	TNF308*2 in Italians COPD and bronchiectasis patients by PCR-RFLP	- TNF gene complex does not seem to play a major role as genetic risk factor in COPD and bronchiectasis.
Yucesoy et al	TNF-308,-238, -376 in Caucasians Silicosis patients by PCR-RFLP	- TNF-α -238 was markedly higher for severe silicosis (4.0) - significantly lower for moderate silicosis (0.52) - TNF-α -308 also showed a strong relationship with disease
Corbett et al	TNF-308,-238, -376 in Black South African Silicosis patients by PCR-SSOP	- no significant differences between miners with less severe silicosis and ctrl. - miners with severe silicosis were significantly more likely than controls to have -238A (33% versus 6%, Fisher's exact p value = 0.022) and -376A (33% versus 5%, Fisher's exact p value = 0.016). - severe silicosis were also significantly more likely to have the -308A allele (Fisher's exact p value = 0.034)

Gene Amplification using PCR

The polymerase chain reaction was developed by Kary Mullis in 1988. Provided that some sequence of the DNA molecules increases exponential quantity of DNA can be obtained from a small number of initial template copies. For example, a single DNA molecule amplified through 30 cycles of replication would theoretically yield 2^{30} (approximately 1 billion) progeny molecules. Single DNA molecule can thus be amplified to yield readily detectable quantities of DNA that can be isolated by molecular cloning or further analyzed directly by restriction endonuclease digestion or nucleotide sequencing.

The starting material is a double-stranded DNA. Large numbers of primers are added, each with the sequence found in one strand at the end of the region to be amplified. The thermostable Taq polymerase and dNTPs are also added.

In the first cycle, heating to 95 °C melts the double-stranded DNA and subsequent cooling to 60 °C then allows the excess primers to hybridize (anneal) to their complementary sequences in the target DNA. The Taq polymerase then extends each primer from its 3′ end by polymerization of dNTPs, generating newly synthesized strands (wavy lines) that extend in the 3′ direction to the 5′ end of the template restriction fragment.

In the second cycle, the original and newly made DNA strands are separated at 95 °C and primers annealed to their complementary sequences at 60 °C. (For simplicity, subsequent events involving only newly made strands are shown; these soon greatly outnumber the original strands.) Each annealed primer again is extended by Taq polymerase to the end of the other primer sequence at the 5′ end of the template strand. Thus the strands (amplimers) synthesized in this cycle exactly equal the length of region to be amplified.

In the third cycle, two double-stranded DNA molecules are generated equal to the sequence of the region to be amplified. These two are doubled in the fourth cycle and are doubled again with each successive cycle. The general procedure for PCR amplification of DNA is illustrated in Figure 4. (101).

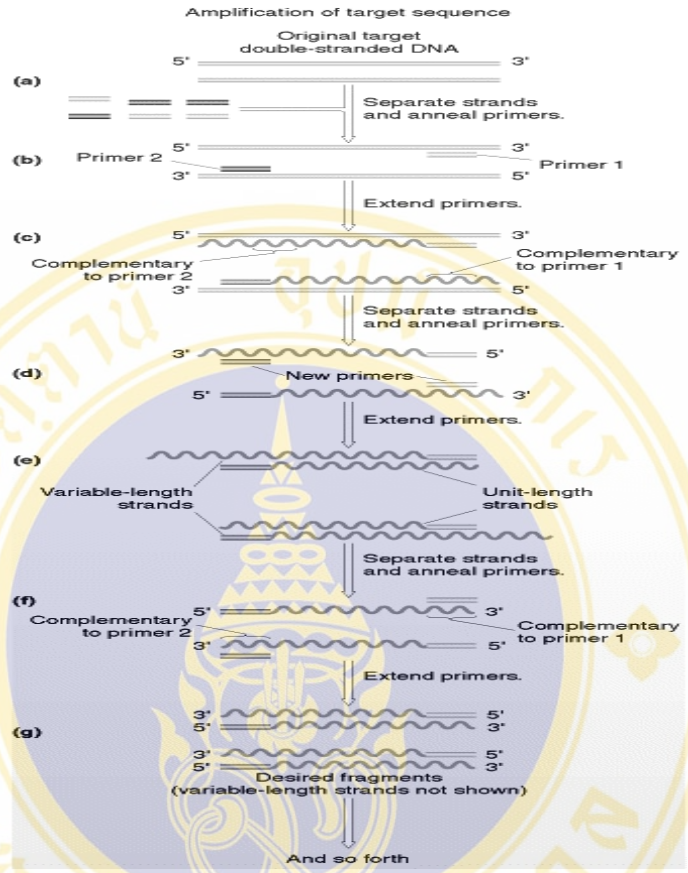

Figure 4 The polymerase chain reaction

The molecular typing of the TNF-α polylmorphisms

PCR-RFLP (polymerase chain reaction- restriction fragment length polymorphism) analysis is base on restriction endonuclease cleavage at polymorphic restriction site. These sited, specific for each enzyme used, are located in both coding and non coding regions of the genes. The technique is not now so widely used in routine laboratories. This is largely due to the dependence on radioactivity for labeling probes; inherent time constraints; difficulties in interpreting result, especially in non-Caucasian populations; the detection of irrelevant polymorphism; the inability to make distinctions between limited nucleotide differences.

PCR-SSOP (polymerase chain reaction – Sequence specific oligonucleotide probe) typing involves PCR amplification of a chosen sequence using primers flanking that sequence. The amplified DNA is immobilized on a nylon membrane and hybridized with selected oligonucleotide probes labeled with either radioactive S35 or P32, or by chemicals.

PCR-SSP (polymerase chain reaction – Sequence specific primers) has mostly been developed by Olerup. In this technique, oligonucleotide primers are designed to obtain amplification of specific alleles or groups of alleles. The typing method is based on the principle that a completely matched primer will be more efficiently used in the PCR reaction than a primer with one or more mismatches. This means that the specificity of the typing system is part of the PCR reaction. Assignment of alleles is based on the presence or absence of amplified products, normally detected by agarose gel electrophoresis and transillumination.

This technique has a number of merits over other PCR based typing methods. It has a high degree of resolution, with each primers pair defining two linked, cis-located polymorphic sites, which facilitates the typing of heterozygous individuals; the PCR-SSP typing are very easy to interpret; the inefficiency of the Taq polymerase to extend mismatched primers is a more precise chemical reaction than the hybridization with ASOs; the post amplification analysis is rapid and more simple than other PCR based methods, because the typing specificity is part of the PCR reaction; the technique is inexpensive; and finally it is very versatile.

PCR-SSP

A polymerase chain reaction with sequence–specific primers (PCR-SSP) methodology is based on the principle that completely matched oligonucleotide primers are more efficiently used in amplifying a target sequence than a mismatched oligonucleotide primer by recombinant Taq polymerase. Primer pairs are designed to have perfect matches only with a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly matched primer pairs result in the amplification of target sequence (i.e., a positive result), while mismatched primer pairs do not result in amplification (i.e., a negative result).

After the PCR process, the amplified DNA fragments are separated by agarose gel electrophoresis and visualized by staining with ethidium bromide and exposure to ultraviolet light. Interpretation of PCR-SSP result is based on the presence or absence of a specific amplified DNA fragment. Since amplification during the PCR reaction may be adversely affected by various factors (pipetting errors, poor DNA quality, presence of inhibitors, etc.), an internal control primer pair is included in every PCR reaction.

The control primer pair amplifies a conserved region of the Human β-globin gene, which is present in all human DNA samples and is used to verify the integrity of the PCR reaction. In the presence of a positive typing band (specific amplification of an HLA allele), the product of the internal control primer pair may be weak or absent due to the differences in concentration and melting temperatures between the specific primer pairs and the internal control primer pair. The amplified DNA fragments of the specific HLA primer pairs are smaller than the product of the internal control primer pair, but larger than the diffuse, unincorporated primer band. Thus, a positive reaction for a specific HLA allele or allele group is visualized on the gel as an amplified DNA fragment between the internal control product band and the unincorporated primer band.

Quality Control for PCR-SSP

It is essential that all the PCR reagents and consumables are tested for efficiency before routine typing commences. The PCR buffer and its key ingredients such as dNTP's, magnessium chloride and Taq polymerase should be tested for optimal concentration. Some Taq polymerases are more efficient than others so it is important to find an optimal Taq concentration. The ratio of dNTP to magnesium chloride is critical and it is advisable to freeze aliquots of magnesium chloride solution rather than store on the shelf as the magnesium will go off over time.

All primer mixes should be batch tested and stored frozen in suitably sized aliquots. Where possible primer mixes should be tested with both positive and negative samples as well as a no-DNA control for PCR contamination. Periodically all PCR reagents should be tested for contamination: If DNA or PCR amplicon contamination is suspected the reagent must be discarded.

To avoid PCR contamination it is recommended that DNA preparation and pre-PCR steps are performed in a different room to post-PCR manipulation. No laboratory equipment should be moved from the post-PCR room to the pre-PCR room. Obviously gloves used in post-PCR steps should be removed on leaving the post-PCR room. If bench-swab PCR contamination tests are performed in the pre-PCR rooms you must make sure that the swab method has a suitable control for PCR inhibitors.

Resolving PCR-SSP problems

1. All reactions have failed (no allele, no control-specific amplicons)

This may be because of either poor quality or insufficient DNA: Test another DNA sample previously shown to work to test reagents and PCR machine. If poor quality DNA is suspected using less DNA with 50% more Taq may work. If it looks like there is lots of DNA by gel electrophoresis then it could be heparin or protein contamination. If heparin contamination is suspected use the heparinase protocol. If protein contamination is suspected try re-extracting by adding 20% v/v 6M NaCl to the remaining DNA and an equal volume of chloroform, vortex, centrifuge at high speed in a microfuge for 5 minutes, extract the aqueous DNA phase and ethanol precipitate as usual.

If a DNA sample shown to previously work starts failing, it is possible that one of the PCR ingredients is faulty or that the DNA sample is degrading over time. Always keep a batch of working frozen stock ingredients so that trouble shooting can be made easier. Fluctuations can be due to variation in Taq supply.

2. Generally weak reactions

Usually due to insufficient or poor quality DNA. Try adding more DNA: if this does not work see the above section on failures.

Incorrectly made buffer or poor/dilute Taq. Remake buffer or try increasing Taq concentrations. Some laboratories use twice or three times the Taq concentration that use.

Inefficient PCR machine: Not all PCR machines work well for this PCR-SSP protocol. If reactions are always weak try elongating some of the PCR program sections or try lower annealing temperatures at the start of the program (68°C instead of 70°C).

3. Too many allele-specific amplicons in one locus

Possible new allele: Try to confirm by sequencing.

Sample contaminated with another DNA sample: Most such contamination would yield extra bands at all loci tested but it is possible to get a combination of alleles in two samples so that the contamination was only noticed at one locus. Most accidental contamination involves small amounts of contaminant being introduced to a larger amount so contaminating bands are typically weak but consistent.

Sample contaminated with a locus-specific amplicon from another part of the laboratory: try to minimize contamination by maintaining good laboratory procedures and spatial separation of pre and post-amplification areas.

Incorrectly made up or contaminated primer mix: Retest suspected primer mix and re-synthesize primer mix if faulty.

4. Too many allele-specific amplicons in all loci

PCR machine error: If the PCR program is interrupted and re-started (especially at the early stages) multiple bands are seen due to the low stringency PCR induced. Use a PCR machine which gives error messages when programs have been interrupted.

Sample or PCR buffer contamination. Remake solutions if contaminated.

5. No allele-specific amplicons at one locus

Homozygous example of a new allele not detected by the given PCR reactions. This is unlikely.

Incorrectly made primer mixes: ensure all primer mixes are tested before use.

Incorrect buffer mixture: If the dNTP to $MgCl_2$ ratio is incorrect it can effect one locus more than another so that it appears as if there are no alleles at one particular locus. Classically false-negative allele amplifications due to incorrect $MgCl_2$ concentrations are associated with much stronger control amplicons and much weaker than normal allele-specific amplicons.

6. Individual reaction failure

Approximately 0.5-1% of PCR-SSP reactions spontaneously fail for no apparent reason. Possible causes include: incomplete PCR reaction, PCR inhibitory contaminant in an individual well, or failure of individual PCR vessels. If a reaction has failed and no primer or primer-dimer is visible on the gel it is likely that either the agarose well was incomplete or the reaction was not loaded into the gel properly.

7. Allele-specific bands present but no controls

Degraded DNA may produce only small amplicons such as the allele-specific amplicons but not larger amplicons such as the control amplicon.

Insufficient PCR extension time: try increasing the time the PCR program spends at the extension temperature (72°C).

PCR machine needs re-calibration.

Concentration of control primers is too low.

8. Control-specific amplicons but no alleles

Magnesium concentration too high: re-calibrate TDMH.

PCR program is inefficient. Try different PCR programs.

Poor fit of PCR tubes/plate into PCR block: The bottom of the PCR vessel must be in direct contact with the PCR block otherwise the correct temperature will not be applied to the PCR reactions. If the fit is suspect dip the vessels in a little light paraffin oil to coat the exterior of the vessel before placing in the PCR machine.

Insufficient pressure from above. If pressure is not applied to the PCR plate the plate may lift out of the block slightly or the thermoseal may peel off. Either way you end up with different PCR thermodynamics which can produce allele drop-out.

Primer mix inefficient at PCR temperature: Some primer mixes do not perform as well as others under the same PCR conditions and this is frequently due to the primer mix being required to work at a slightly inappropriate temperature. Check the primer mix at different PCR temperatures to see if the primer mix works at a different temperature: this can be done using several PCR machines, or more conveniently with a PCR machine with a gradient block such as the Eppendorf Mastercycler Gradient PCR machine. If a primer mixes works at a temperature lower than that required by the rest of the primer mixes in the set one or both of the primers will have to be lengthened by one or two bases to increase the primer(s) annealing temperature. If a primer pair works at a hotter temperature it indicates there is a structural problem with one of the primers (such as primer sequence tending to form hairpin loops). If a structural problem is suspected it is recommended that the primer mixes are redesigned or that the primer concentration is radically increased.

9. Part of the typing has worked well, but the remainder has failed

PCR machine failure. This is a common failure if a PCR machine is used intensively. Test blocks uniformity by amplifying 96 identical reactions in one plate. If a problem does exist contact a PCR service engineer.

PCR plate not placed in machine properly.

Uneven pressure applied during PCR.

Gel artifact caused by insufficient ethidium bromide

CHAPER IV

MATERIALS AND METHODS

Sample size

With the expectation that TNF-α -308*2 allele frequency in patient group will be 20% while in control group from previous data was 5%, the sample size will be 82 patients in each group for 95% confidence level and 80% power of test. Calculated by SAM: A sample calculator program. (NHNIC Clinical Trial Centre, 1991).

Study design

Case- control study

Subject

Two Case groups were used in the study:

1. The COPD group consisted of smoking related COPD patients (at least 15 pack per years) recruited from the Division of Respiratory Disease and Tuberculosis of Siriraj Hospital. COPD was diagnosed on the basis of past history, physical examination and spirometric data, according to American Thoracic Society guidelines (74). Spirometry were performed to determine forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1), using a standard spirometer (Compact II, Vitalograph, Buckingham, England), in a sitting position. Chronic airflow obstruction was defined by FEV1/FVC < 90% and FEV1 < 80% of predicted values. Subjects were excluded if they had a history of asthma.

2. The Silicosis group (recruited from the same unit) consisted of a patient with 2 from 3 criterion

1. A person's occupational history with silica dust exposure at least 5 years mostly from related rock-grinding industries

2. A chest X-ray characteristic according to the ILO (International Classification of Radiographs of Pneumoconiosis 1980) standard.

3. Characteristic lung tissue pathology of silicosis.

Two control groups were used in the study:

1. A smoker control group that included asymptomatic smokers and ex-smokers matched for sex and age with a smoking history of at least 15 pack per years but without COPD and asthma recruited from the Division of Respiratory Disease and Tuberculosis of Siriraj Hospital. They had normal pulmonary function (FEV1/FVC ≥ 90% and FEV1 >80% of predicted values)

2. A population control group of adult Thai blood donors from Department of Transfusion Medicine, Siriraj Hospital.

The Research Ethics Committee of Faculty of Graduate Studies, Mahidol University approved the study, and all subjects gave their informed consent in writing.

Methods

The biallelic polymorphisms within TNF-α is between (~) 70 bp and 700 bp apart, making a PCR-SSP system with 3' mismatch in the forward and reverse primers feasible. The advantage of this strategy is that polymorphisms are physically linked through PCR and haplotypes can be determined unequivocally without the use of families. The logically extension of this concept would be to link polymorphic sites in adjacent genes. A TNF genotyping system based on the outlined strategy would serve as model for PCR-haplotyping of other genes, particularly those in the MHC regions.

DNA isolation and TNF-∝ gene polymorphism typing

DNA derived from peripheral blood leukocytes and isolated by Guanidine-HCl method from UCLA Tissue Typing Laboratory, 1993 (Appendix B). The amplified DNA segments were analyzed by PCR-SSP (Polymerase chain reaction- Specific sequence primers) using TNF primers designed to detect the G or A variant of the 4 polymorphism in the promoter region.

PCR primer design

The TNF sequence GenBank: Z15026 (102) was used to generate the primers. Mismatches at the 3'end in both the forward and reverse primers were including (103). The primers were combined to link polymorphic sites within TNF and are listed in Tables 2 and 3.

PCR cycling conditions

PCR amplifications were carried out in Perkin Elmer 9600 machines under the following cycling conditions: 2 min at 96°C; 5 cycles for 25s at 96°C, 45s at 70°C, 45s at 72°C; 21 cycles of 25s at 96°C, 50s at 65°C and 45s at 72°C; 4 cycles of 25s at 96°C, 60s at 55°C and 120s at 72°C.

PCR preparation

PCR was carried out in a final volume of 13 µl in 96- or 192-well plates under conditions identical to those described for HLA phototyping (104). Primer mixes (5 µl) overlaid with 10 µl of mineral oil was prepared in advanced and stored at –20ºC until use. The final concentration of reaction components were as follows: 200 µM of each dNTP, primers (as specified in Table 1), 2 mM $MgCl_2$, 67 mM Tris Base pH 8.8, 16.6 mM ammonium sulfate, 0.01% (v/v) Tween 20, between 0.01 and 0.1 µg of DNA and 0.2 units of Taq polymerase (Advanced Biotechnology,London, UK). Each plate was centrifuged briefly (20 s at 500 rpm) before being placed into a PCR machine.

Table 2 Oligonucleotides designed to detect polymophisms in TNF-α. Note primers 708 and 709 correspond to primers c2 and c3 described by Verjans et al (105). The control primers 63, 64, 210 and 211 were specific for exon 3 sequences in HLA-B, which were used as controls for HLA phototyping

	TNF primers	Working concentration (μM)
644	5-CTC CCC ATC CTC CCT gCT CC	0.49
645	5-CTC CCC ATC CTC CCT gCT CT	0.62
708	5-ATA ggT TTT gAg ggg CAT Cg	0.56
709	5-ATA ggT TTT gAg ggg CAT CA	0.56
710	5-gCA TCC CCg TCT TTC TCC AC	0.58
711	5-gCA TCC CCg TCT TTC TCC AT	0.47
712	5-gAA gCA CCC CCT Cgg AAT Cg	0.57
713	5-gAA gCA CCC CCT Cgg AAT CA	0.57
63	5-TgC CAA gTg CAC CCA A	0.1
64	5-gCA TCT TgC TCT gTg CAg AT	0.1
210	5-ATg ATg TTg ACC TTT CCA ggg	2.0
211	5-TTC TgT AAC TTT TCA TCA gTT gC	2.0

Table 3 The 12 combinations of primers specific for each polymorphism combination listed. Each reaction also contained control PCR primers to avoid false negative interpretations. Reactions 1-8 included control primers 210-211 (256 bp) and 9-12 included control primers 63-64 (796 bp). The use of large and small control amplicons made haplotype-specific positive reactions easy to identify under the gel electrophoresis conditions described

		TNF			
Primer mixes		488	-238	-308	Size of amplicon (bp)
1	710-708	g		g	835
2	710-709	g		a	
3	711-708	a		g	
4	711-709	a		a	
5	710-713	g	a		763
6	710-712	g	g		
7	711-713	a	a		
8	711-712	a	g		
9	644-708		g	g	109
10	645-708		a	g	
11	644-709		g	a	
12	645-709		a	a	

Electrophoresis

The entire PCR reaction (13 µl) plus 10 µl of loading dye were loaded into a 1% agarose gel (containing ethidium bromide) in 1x TBE. Gels were electrophored for 25 min at 200 V/cm^2. The gels were photographed (b/w) under ultraviolet light (320 nm).

Interpretation

The specificity of each reaction is listed in Table 2, and the gel is scored for the presence or absence of an allele-specific band, providing that a PCR control band was present. Note that the first 8 reactions have a small control band (256 bp) and the remaining 16 have a large control band (796 bp). The decision to use the 256-bp control amplicon in reactions 1-8 and the 796-bp control amplicon in reactions 9-24 was based on the ease of gel interpretation after 25 min of electrophoresis (200 mV) on 1% agarose gel.

Nomenclature for TNF

With reference, the haplotypes for TNF-α were assigned separately and were used to name alleles for each locus based on the frequencies observed in United Kingdom populations. The most frequent TNF-α allele was denoted TNF 1, the next TNF 2 etc. This system made reporting haplotype frequencies easier in this publication

Statistical analysis

The allele frequencies were determined by direct counting, and gene frequencies were calculated using Eq.1. (12). The X^2- test was used to detect significant differences in TNF-α gene polymorphism. P-values smaller than 0.05 were taken as significantly different.

Eq.1. $p = 1-\sqrt{1-f}$

p = gene frequency, f = allele frequency calculated by direct counting

CHAPTER V

RESULTS

Using the method described by TNF sequence GenBank: Z15026 (102). The 12-reaction TNF-α set was designed to detect all eight possible haplotypes of three biallelic polymorphisms. Of these possible haplotypes, 4 were observed in these studies (Table 4).

Table 4 Haplotypes of TNF-∝ in Thailand

Position	+488	-238	-308
TNF1	G	G	G
TNF2	G	G	A
TNF3	A	G	G
TNF4	G	A	G

The 12 combinations of primers specific for each polymorphism, each reaction also contained control PCR primers to avoid false negative interpretations. Reactions 1-8 included control primers 210-211 (256 bp) and 9-12 included control primers 63-64 (796 bp). The use of large and small control amplicons made haplotype-specific positive reactions easy to identify under the gel electrophoresis conditions described. The final PCR-SSP products from the electrophoresis and were photographed (b/w) under ultraviolet light (320 nm) were illustration in figure 5-8.

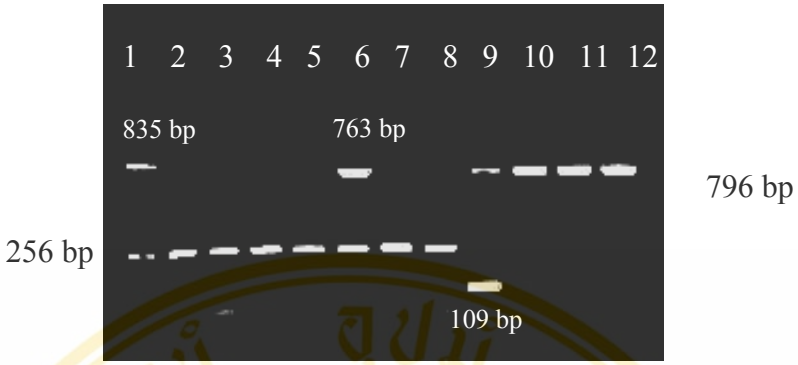

Figure 5 The final PCR-SSP products of TNF 1 phenotype from the electrophoresis and were photographed (b/w) under ultraviolet light (320 nm).

The lane numbers 1, 6 and 9 are equivalent to the reaction numbers shown in table 2 and in Figure 1.

The use of small controls (256 bp) in reactions 1-8 and large controls (796 bp) in reactions 9-12.

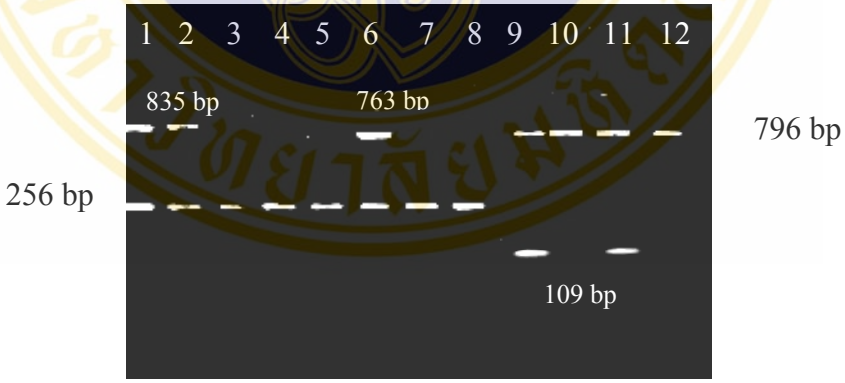

Figure 6 The final PCR-SSP products of TNF2 from the electrophoresis and were photographed (b/w) under ultraviolet light (320 nm).

The lane numbers 1, 2, 6, 9 and 11 are equivalent to the reaction numbers shown in table 2 and in Figure 1.

The use of small controls (256 bp) in reactions 1-8 and large controls (796 bp) in reactions 9-12.

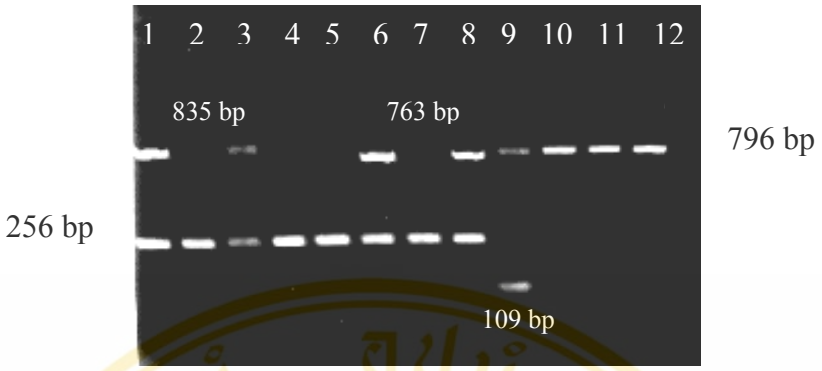

Figure 7 The final PCR-SSP products of TNF3 from the electrophoresis and were photographed (b/w) under ultraviolet light (320 nm).

The lane numbers 1, 3, 6, 8 and 9 are equivalent to the reaction numbers shown in table 2 and in Figure 1.

The use of small controls (256 bp) in reactions 1-8 and large controls (796 bp) in reactions 9-12.

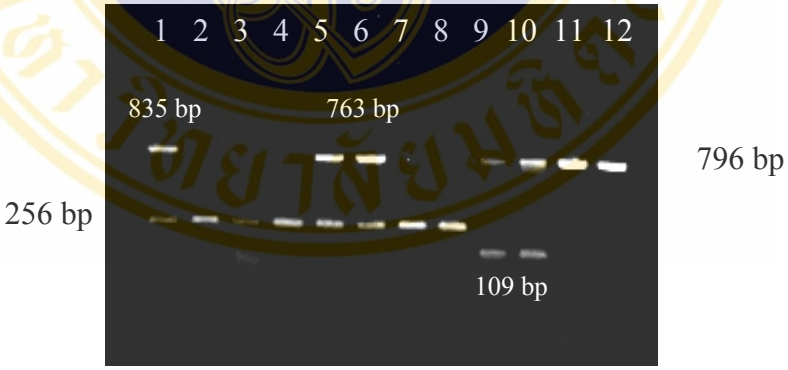

Figure 8 The final PCR-SSP products of TNF4 from the electrophoresis and were photographed (b/w) under ultraviolet light (320 nm).

The lane numbers 1, 5, 6, 9 and 10 are equivalent to the reaction numbers shown in table 2 and in Figure 1.

The use of small controls (256 bp) in reactions 1-8 and large controls (796 bp) in reactions 9-12.

The frequencies of TNF-α polymorphisms were studies in 82 COPD and 48 silicosis patients. In COPD group, the frequencies were compared with 82 smokers but without COPD and asthma. 117 anonymous blood donors were used as populations control in each group.

The gene frequencies at the individual loci were compared with published frequencies (Table 5). For position TNF +488 and -238 the observed allele frequencies for the Thai were similar to the frequencies published by Fanning et al. for United Kingdom population (12). For position -308G/A allele, the observed allele frequencies were significant difference to the frequencies of caucasian but not difference to the frequencies of Japanese (7): in Thai population, the -308G/A allele frequencies were 0.95/0.05; in United Kingdom population, 0.79/0.21 (P-value ≤0.01) and in Japanese population, (0.92/0.08).

Table 5 Individual TNF-α biallelic polymorphism frequency in 117 Thai population controls. United Kingdom frequencies were taken from Fanning et al. and Japanese frequencies were taken from Sakao et al.

		Thai (n = 234)		UK (n = 402)		Japanese (n=258)	
TNF	Nucleotide	n	%	n	%	n	%
+ 488	G	219	0.94	362	0.90	ND	
	A	15	0.06	40	0.10	ND	
- 238	G	228	0.97	378	0.94	ND	
	A	6	0.03	24	0.06	ND	
- 308	G	223	0.95	318	0.79	238	0.92
	A	11	0.05	84	0.21	20	0.08

The haplotype frequencies of these studies were summarized in table 6. The TNF1 haplotype were detected in 81.1% of COPD patients, 79.88% of smoker control, 80.21 % of silicosis patients and 86.32% of healthy control. TNF2 haplotype were detected in 7.93% of COPD patients, 5.49% of smoker control, 12.5% of silicosis patients and 4.7% of healthy control. TNF3 haplotype were detected in 9.76% of COPD patients as same as smoker control, 5.21% of silicosis patients and 6.41% of healthy control. TNF4 haplotype were detected in 1.22% of COPD patients, 4.88% of smoker control, 2.08 % of silicosis patients and 2.56% of healthy control.

Table 6 Haplotype count for TNF-α

	Haplotypes count			
	TNF1 (%)	TNF2 (%)	TNF3 (%)	TNF4 (%)
COPD (n=82)	133 (81.1)	13 (7.93)	16 (9.76)	2 (1.22)
Smoker control (n=82)	131 (79.88)	9 (5.49)	16 (9.76)	8 (4.88)
Silicosis (n=48)	77 (80.21)	12 (12.5)	5 (5.21)	2 (2.08)
Population control (n=117)	202 (86.32)	11 (4.7)	15 (6.41)	6 (2.56)

Table 7 shows the frequency of TNF-α biallelic polymorphism at the individual loci. By Yates corrected Chi-squares test, there were no significant difference in the distribution of TNF-α allele among patients with COPD, smoker control subjects, and population control subjects. But the distribution of position –308G/A allele frequencies were significantly different between silicosis patients and population control subjects (p-value≤0.05). The –308G/A allele frequencies of silicosis patients were 0.88/0.12 and population controls were 0.95/0.05. (Odds ratio = 2.9). The statistical analysis of TNF-α polymorphisms were summarized in Table 8.

Table 7 TNF-α biallelic polymorphism frequency. Gene frequencies were determined using the formula $p = 1-\sqrt{1-f}$ (f = allele frequency)

TNF	Nucleotide	Allele count	Allele frequency	Gene frequency
COPD (n=164)				
+ 488	G	148	0.90	0.68
	A	16	0.10	0.05
- 238	G	162	0.99	0.90
	A	2	0.01	0.01
- 308	G	151	0.92	0.72
	A	13	0.08	0.04
Smoker control (n=164)				
+ 488	G	148	0.90	0.68
	A	16	0.10	0.05
- 238	G	156	0.95	0.78
	A	8	0.05	0.03
- 308	G	155	0.95	0.78
	A	9	0.05	0.03
Silicosis (n=96)				
+ 488	G	91	0.95	0.78
	A	5	0.05	0.03
- 238	G	94	0.98	0.86
	A	2	0.02	0.01
- 308	G	84	0.88	0.65
	A	12	0.12	0.06
Population control (n = 234)				
+ 488	G	219	0.94	0.75
	A	15	0.06	0.03
- 238	G	228	0.97	0.84
	A	6	0.03	0.01
- 308	G	223	0.95	0.78
	A	11	0.05	0.03

Table 8 The statistical analysis of TNF-α between COPD, silicosis and control

	Odd ratio	Relative risk	Chi-squares	P-values
COPD (n=82) Vs Population control (n=117)				
+488	1.58	1.52	1.07	0.30
-238	0.47	0.48	0.33	0.56
-308	1.75	1.69	1.25	0.26
COPD (n=82) Vs Smoker control (n=82)				
+488	1.00	1.00	0.03	0.85
-238	0.24	0.25	2.58	0.11
-308	1.48	1.44	0.44	0.51
Silicosis (n=46) Vs Population control (n=117)				
+488	0.80	0.81	0.03	0.87
-238	0.81	0.81	0.02	0.89
-308	2.9	2.67	5.24	0.02

The genotyping frequencies of TNF-α gene promoter in these studies were illustration in Table 9. The most common genotype of TNF-α gene promoter was GG. , the rarer genotype was AA.

Table 9 Genotype frequency of TNF-α gene promoter. Black South African genotype frequencies were taken from Corbett et al (100).

	+488	-238	-308
	Count (%)	Count (%)	Count (%)
COPD (n=82)			
GG	67 (82)	80 (98)	69 (84)
GA	14 (17)	2 (2)	13 (16)
AA	1 (1)	0	0
Combined AA/AG (%)	18	2	16
Smoker control (n=82)			
GG	66 (80)	74 (90)	73 (89)
GA	16 (20)	8 (10)	9 (11)
AA	0	0	0
Combined AA/AG (%)	20	10	11
Population control (n=117)			
GG	103 (88)	111 (95)	73 (89)
GA	13 (11)	6 (5)	9 (11)
AA	1 (1)	0	0
Combined AA/AG (%)	12	5	11
Silicosis (n=48)			
GG	43 (90)	46 (96)	36 (75)
GA	5 (10)	2 (4)	12 (25)
AA	0	0	0
Combined AA/AG (%)	10	4	25
Black South African population (n=120)			
GG	ND	113 (94)	73 (61)
GA	ND	5 (4)	36 (30)
AA	ND	2 (2)	11 (9)
Combined AA/AG (%)	ND	6	39

By Yates corrected X^2 test, there were no significant difference in the distribution of TNF-α genotype frequency among patients with COPD, smoker control subjects, and population control subjects. But the distribution of position –308G/A genotype frequencies were significantly different between silicosis patients and population control subjects (p=0.02). The –308G/A genotype frequencies of silicosis patients were 0.75/0.25 and population controls were 0.89/0.11 (Odds ratio = 2.7). Genotype frequency between Thai and Black South African normal population were significant difference in position -308 (p-value ≤0.01). The statistical analysis of TNF-α polymorphisms were summarized in Table 10.

Table10 The statistical analysis of TNF-α genotype frequency

	Odd ratio	Relative risk	Chi-squares	P-values
COPD (n=82) Vs Population control (n=117)				
+488	0.53	0.92	1.96	0.16
-238	2.58	1.03	0.59	0.44
-308	0.65	0.94	0.69	0.41
COPD (n=82) Vs Smoker control (n=82)				
+488	1.02	1.00	0.01	0.90
-238	5.44	1.09	4.34	0.04
-308	0.94	0.99	0.00	0.97
Silicosis (n=46) Vs Population control (n=117)				
+488	1.23	1.02	0.05	0.82
-238	1.26	1.01	0.00	1.00
-308	2.7	1.19	5.72	0.02
Population control (n=117) Vs Black South African population (n=120)				
+488	ND	ND	ND	ND
-238	1.21	1.01	0.00	1.00
-308	5.17	1.46	19.44	<0.01

CHAPER VI

DISCUSSION

Chronic inflammatory diseases, such as COPD and silicosis are multifactorial, determined by both environmental and genetic factors. While the interaction of genes with environmental and host factors initiates the disease process, other genetic factors, such as polymorphism, can modify the extent of the disease. COPD and silicosis, resulting from the interaction of smoking and silica particles and lung cells, is manifested by persistent overproduction of inflammatory mediators, leading to alveolar damage, fibroblast proliferation, collagen deposition and granuloma formation. Animal models have suggested that IL-1 and, particularly, TNF-α play significant roles in silica-induced lung damage (89, 90, 91). For example, treatment with neutralizing antibodies to TNF-α causes a significant reduction of the fibrotic response in silica-exposed mice (93).

Investigation of the genetic component of multifactorial disorders such as COPD and silicosis were scientific priority. Based on the knowledge, albeit limited, of the biochemical basis of COPD and silicosis, a number of putative candidate genes for this disease may be postulated, and, among them, the TNF gene complex may play a role. The aim of the present study was to evaluate the frequencies of TNF-α gene promoter polymorphisms in Thai patients with COPD and silicosis, and compare them with the frequencies in appropriate control subjects.

The comparison between Thai and Caucasian normal population were significant difference in TNF-α -308 allele (P-value≤ 0.01) (8). But there were similarity between Thai and Japanese normal population (7). The differences in the association between COPD in Asian and white normal populations may not be explained by differences in TNF-α -308 allele frequency between the normal populations. There

seems to be an ethnic difference in the prevalence. This study demonstrated that there were no difference in frequency of the TNF-α gene promoter in Thai patients with COPD, smoker control subjects, and population control subjects.

Previous studies, in Taiwanese populations, TNF-α -308*2 polymorphisms confers a higher risk to the development of chronic bronchitis (6). In Japanese populations, TNF-α -308 1/2 alleles were significantly associated with the presence of smoking-related COPD (7). However, there were no significant differences in TNF1/2 allele frequencies in Caucasian (8).

This suggests that ethnic genetic background is another factor to be considered when results of the two studies are compared. There may also be differences in the prevalence of COPD in different ethnic groups, but these are difficult to separate from lifestyle factors. For example, the prevalence of COPD is apparently low in China and this cannot be entirely accounted for by lower tobacco consumption (106). Anecdotally, COPD is uncommon in Chinese living in the USA which suggests that there may be genetic differences in the factors that protect against COPD. In Hawaii, the prevalence of COPD in Japanese-Americans smoking more than 20 cigarettes daily was 7.9% compared with 16.7% in a matched Caucasian-American group (107). More studies are needed in different ethnic groups, particularly those living overseas, in order to explore these ethnic differences. Differences in the prevalence of COPD in different ethnic groups are likely to be accounted for by the differing frequencies of genes relevant to pathogenesis, so that exploration of these differences at a molecular level may be informative. For example, the ZZ phenotype of α_1-AT does not occur in black subjects and is very rare in Asians, and abnormalities in cystic fibrosis transmembrane regulator (CFTR) do not occur in the Japanese population. These differences in gene frequency between different racial groups make comparisons between different populations difficult and may account for some of the reported differences in the association between gene polymorphisms and COPD in different studies.

Several studies have shown an increased prevalence of COPD within families. Case control studies have demonstrated an increased prevalence of COPD in

relatives of patients with COPD which cannot be accounted for by known risk factors such as smoking (108, 109, 110, 111). Regressive models established to search for genetic factors in patients with COPD suggest major gene effects compared with families without pulmonary diseases (112). In the Framingham study segregation analysis of over 5000 subjects from over 1000 families suggested that, after correction for smoking, polygenic gene effects and other environmental factors determine forced expiratory volume in one second (FEV_1) (113). A recent studies showed that lung function was reduced in first degree relatives of patients with early onset COPD only if they currently or previously smoked, with an increased odds ratio of approximately 3 (114). Genetic influences determine pulmonary function and there is a closer similarity in spirometric measurements between monozygotic (identical) than dizygotic twins (78). Twin studies in smokers have shown that there is a high risk in monozygotic twins that both will develop airflow obstruction, whereas in dizygotic twins who smoke (115), even if raised apart (116), this is not the case.

Previous study demonstrated significant associations between the -238A, -376A and -308A alleles of the TNF-promoter and severe patients of silicosis in Black South African mineworker. There were no significant differences between miners with less severe silicosis and controls at any loci (9). In Caucasian study, TNF-α -238A was markedly higher for severe silicosis (4.0) and significantly lower for moderate silicosis (0.52) (100).

The distribution of TNF-α allele in Thai patients with silicosis and population control subjects were significantly different both in –308A allele frequency ($p<0.05$) and -308G/A genotype frequency ($p=0.02$). The genotype frequency at -308 between Thai and Black South African normal populations were significant differences ($p\leq 0.01$). The ethnic genetic background is another factor to be considered. There may also be differences in the prevalence of silicosis in different ethnic groups.

Polymorphisms in the TNF-α promoter region are unusually strong candidates as putative silicosis-susceptibility loci, since TNF-α has a central causal role in the pathophysiology of acute silicosis (117), and significant associations between silicosis and certain HLA have been reported (118, 119), but with inconsistency of the

implicated antigens in different populations, suggesting linkage disequilibrium with a nearby susceptibility locus, rather than a genuine HLA effect. The TNF-α locus lies within the HLA complex, so that linkage disequilibrium with HLA antigens would be expected among diseases associated with polymorphisms in the TNF-α promoter region (33). The basis for associations between TNF-α promoter polymorphisms and disease susceptibilities is becoming clearer. In some cases SNPs have been associated with functional effects such as binding of nuclear transcription factors, increased basal or inducible transcriptional activity, or increased TNF-α production in vivo (33, 120, 121, 122). The genetics and function of this region, however, remains incompletely determined (123).

In the study of other associated genes with silicosis, a gene in linkage disequilibrium with HLA-Bw54, DR4, and DRw53 might control the susceptibility to silicosis (119). And further define the HLA-linked gene and other genetic factors for predisposition of silicosis, they determined HLA-DQ and DP alleles using the polymerase chain reaction and sequence-specific oligonucleotide probes and made a restriction fragment length polymorphism (RFLP) analysis of the fourth component of complement (C4) genes, immunoglobulin lambda variable chain (IGLV) gene, and T-cell receptor alpha and beta genes in 46 Japanese patients with silicosis. The frequency of DQB1*0401 (relative risk [RR] = 2.2, $P < 0.02$) was increased and that of DQB1*0601 (RR = 0.36, $P < 0.01$) was decreased in the patients. RFLP analysis of C4 and IGLV genes showed a significant association between silicosis and a specific RFLP pattern of C4A3-C4B5 allotype (RR = 2.3, $P < 0.05$) and that of IGLV 5.3 kb (RR = 0.33, $P < 0.003$). None of other genetic markers showed significant association. Statistical analyses of the associated genetic markers revealed that the HLA-Bw54 was the allele that showed primary association with silicosis and the frequencies of the C4 and HLA-DQ alleles were suggested to be increased due to their linkage disequilibrium with the HLA-Bw54. They concluded that the major gene for silicosis may be mapped near the HLA-B locus. Therefore it is possible that there are several candidate genes associated with silicosis.

The present study had several limitations, including small sample size, lack of control and disease severity profiles. In silicosis group, there were no control subjects

without silicosis from the same industries. The severity of disease may be different from other studies. The primary association of a TNF polymorphism with a given condition requires a large sample size, with as many SNPs as possible. The alternative is to study families, where haplotype can be much easier interpreted and other genetic approaches such as the transmission disequilibrium test can be used to check whether frequencies of polymorphism/haplotype is being inherited unambiguously rather than by chance from a heterozygote parents to an affected offspring.

CHAPTER VII

CONCLUSION

Although COPD and silicosis shared some related pathogenesis via TNF-α the role for disease development of TNF-α gene promoter polymorphism, our study demonstrated only the association of silicosis was found with TNF-α -308A allele. But in COPD, association of this allele was not observed.

Previous studies, some populations reported association of TNF-α with COPD (Taiwanese and Japanese were found with TNF-α -308A allele) (6, 7). And some populations found no association at any loci (Caucasian) (8). In silicosis, Black South African miners demonstrated the association of TNF-α -238A, -376A and -308A alleles only in the more severe patients according to the radiographic findings (9). And Caucasian study showed that TNF-α -238A was markedly higher for severe silicosis. The discrepancy between our study and previous study may result from 1) Different in frequency of normal population. 2) Different in clinical severity and prevalence of the disease.

It is difficult to make general statements about the associations of TNF-α polymorphisms and TNF-α production or pathology. The TNF-α polymorphisms are found in a region of great polymorphic variation and they are in linkage disequilibrium with the HLA genes and with each other. Cause of differences in the distribution of HLA alleles one might expect a variation in the association between TNF polymorphisms and various conditions in different geographical areas. Dissecting out a primary association with a polymorphism within the TNF-α locus could be tricky.

The potential limitations of this study resulted from small sample size. In silicosis group, there was no control subject from the same industries but without silicosis and there was no data of severe cases.

Unsolved questions

1. The TNF-α gene is consisted of 7 polymorphic sites (12). In this study we evaluate only three well established TNF polymorphisms +488, -238 and –308. Our results showed COPD patients did not exhibit the TNF-α polymorphisms at these three common sites. It is possible that polymorphisms in TNF-α gene may occur at the remaining in four polymorphic positions. This is required to be further determined.

2. Previous study showed female sex hormones may play a protective role against insults that injure lung tissues. It was demonstrated that TNF-α production by peripheral blood lymphocytes varied considerably in premenopausal females, while it was more constant in men and postmenopausal females (57), suggesting that one of the significant risk factors for COPD is male sex. We will further demonstrate that gender affects TNF-α polymorphism difference between male and female.

3. COPD patients who have these TNF-α gene polymorphisms will probably get more severity than other patients and the progress of disease will be more rapid. It is interesting in investigating whether those who had their lung function determination, especially severe COPD would have TNF-α gene polymorphisms.

Future direction

What we need to do further is as follows

1. Increase number of sample and control
2. Demographic of patient
3. Appropriate control
4. Other polymorphisms

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APPENDIX

APPENDIX A

REAGENT

Sample preparation

5% EDTA for whole blood pH 7.4

EDTA (di-Sodium Salt)	25	gm.
dd H_2O	450	ml.
Adjust pH 7.4		
Add dd H_2O up to	500	ml.
Autoclaved		

DNA preparation

Solution A

NH_4Cl	25.4	gm.
EDTA	5.32	gm.
Trisma Base	3.68	gm.
dd H_2O	3600	ml.
Adjust pH 7.2		
Add dd H_2O up to	4000	ml.
Autoclaved		

7.5 M Guanidine- HCl

Guanidine- HCl	432	gm.
1 M Tris- HCl (pH 7.6)	60	ml.
add dd H_2O (Autoclaved) up to	600	ml.
Filter with 0.2 um		
Autoclaved		

1 M TRIS-HCl pH 7.6

Tris-HCl	121.1	gm.
dd H_2O	900	ml.
adjust pH to 7.6 with conc. HCl		
add dd H_2O up to	1000	ml.
autoclaved		

10% w/v sodium dodecyl sulphate (SDS)

SDS	100	gm.
add ddH_2O up to	1000	ml.

10 mg/ml Proteinase K

Proteinase K	100	mg.
add dd H_2O up to	10	ml.

80% Ethanol

absolute ethanol	800	ml.
add ddH_2O up to	1000	ml.

TE BUFFER pH 8.0

Tris-HCl	1.211	gm.
EDTA	0.037	gm.
dd H_2O	950	ml.
adjust pH 8.0		
add dd H_2O up to	1000	ml.
Filter with	0.45	µm.
autoclaved		

PCR Ingredients

10X PCR BUFFER

25 mM $MgCl_2$	10.4	ml.
1 M Tris-HCl pH 8.8	6.96	ml.
1.5 M Ammonium sulfate	1.12	ml.
1% Tween 20	1.04	ml.
ddH_2O to	24.0	ml.
store at -20 ^oC		
ddH_2O	5520	µl.
aliquot and store at -20 ^oC		

1 M $MgCl_2$

$MgCl_26H_2O$	203.3	gm.
add ddH_2O to	1000	ml.
autoclaved		

1 M TRIS-HCl pH 8.8

Tris-HCl	121.1	gm.
dd H_2O	900.0	ml.
adjust pH to 8.8 with conc. HCl		
add dd H_2O up to	1000	ml.
autoclaved		

1.5 mM Ammonium sulfate

Ammonium sulfate	1.982	gm
add autoclaved dd H_2O up to	10	ml.

1% (v/v) Tween 20

tween 20	1	ml.
add ddH_2O up to	100	ml.

2 mM dNTP

100 mM dATP	120	µl.
100 mM dCTP	120	µl.
100 mM dGTP	120	µl.
100 mM dTTP	120	µl.

Gel electrophoresis

10X TBE Buffer

TBE buffer dry powder	1	bottle
ddH_2O up to	4000	ml.
Filter with	0.2	µm.
Autoclaved		

1% Agarose gel (For small chamber)

Agarose powder type II	0.75	gm.
1X TBE	75	ml.
Microwave		
add 5 mg/ml Ethidium bromide	7.5	µl.

5 mg/ml Ethidium bromide

Ethidium bromide	50	mg.
ddH_2O	10	ml.

Loading buffer

50 mM Tris-HCl pH 7.6	1 M Tris-HCl pH 7.6	0.5	ml.
50mM EDTA pH 8.0	1.5 EDTA pH 8.0	1.0	ml.
0.5 % SDS	10 % SDS	0.5	ml.
0.1 % w/v Bromphenol Blue	10 %BP blue	0.1	ml.
40 % Sucrose	sucrose	4.0	gm.
add ddH_2O up to		10	ml.

1M Tris-HCl pH 7.6

Tris-HCl	121.1	gm.
ddH_2O	900	ml.
adjust pH to 7.6 with conc. HCl		
add ddH_2O up to	1000	ml.
Autoclaved		

10% w/v Bromphenol Blue

Bromphenol Blue	1	gm.
add ddH_2O up to	10	ml.

Buffer for electrophoresis (1X TBE)

10X TBE	100	ml.
0.5 ug/ml Ethidium bromide ddH_2O up to	1000	ml.
5 mg/ml Ethidium bromide	100	µl.

Marker

Phi X 174 Hae III fragment	20 ul. of 0.7 ug/ul Stock Solution	
dd H_2O	150	µl.
Loading Buffer	30	µl.
3.5 ug/ml. Solution of marker		

APPENDIX B

DNA extraction using Guanidine-HCl extraction (from UCLA Tissue Typing Laboratory, 1993.)

1. Centrifuge the tube(s) of whole blood at 1,800 rpm for 10 minutes.

2. Transfer 250-500 ul of buffy coat or ficoll pellet to a 1.5 ml Eppendorf tube.

3. Add 1.0 ml of Solution A or other RBC-lysing reagent.

   Vortex and stand for 2 minutes. Spin at 5,000 rpm or 2,000 g for 1 minute.

   Discard the supernatant.

   (Repeat this step until all RBCs are lysed however, does not repeat this step more than 3times.)

4. Vortex the pellet to prevent clumping. Approximate the pellet size and add the appropriate volumes of reagents as listed in the following chart.

Pellet size	100-50 ul	50-25 ul	25-10 ul
Proteinase K	40 ul	20 ul	12 ul
ddH_2O	800 ul	400 ul	300 ul
10% SDS	300 ul	150 ul	105 ul
7.5M Guan.HCl	300 ul	150 ul	105 ul

Ethanol Precipetation

Ethanol	4.0 ml	2.0 ml	1.0 ml
Labeled tube	15 ml Falcon	15 ml Falcon	1.5ml Falcon

5. Add Proteinase K. Vortex the sample.

6. Add ddH_2O. Vortex the sample again.

7. Add 10% SDS. Mix the sample gentle by rocking the tube back-and -forth.

8. Add 7.5M Guanidine HCl. Again, mix the sample gently.

9. Incubate the sample at 68-70°C for 10 minutes.

10. After the 10 minutes, mix the sample vigorously using pipettes until the mixture becomes homogeneous. Try to avoid creating bubbles while mixing with pipettes.

11. Incubate the sample at 68-70°C for 10 minutes.

12. After the 10 minutes, spin the sample at 14,000g for 4 minutes at 4^{o}C

13. **a**. If the pellet is compact and the supernatant is clear and free of debris, continue to the next step.

b. If the pellet is diffuse and the supernatant is cloudy, repeat steps 9-12

14. Transfer the supernatant to the appropriate labeled tube by decanting or pipetting. Slowly and appropriate volume of Ethanol to maintain the interface between the two phases. Gently rock the tube back-and forth until cotton-like strands of DNA appear.

15. Vortex the sample to tighten the pellet. Transfer the DNA to another labeled 1.5 ml Eppendorf tube by drawing 800 ul of DNA-EtOH using a blue pipette-tip.

16. Spin at 10,000 g for 2 minutes. Discard the alcohol supernatant.

17. Add 500 ul of 80% ethanol to the sample, vortex to loosen the pellet and let the sample stand for 1 minute.

18. Spin the sample at 10,000 g for 2 minutes. Discard as much of the supernatant as possible.

19. Add 200 ul of ddH_2O or TE buffer pH 8.0 to the sample. Vortex and incubate at 68-70°C for 10 minutes with the cap open to evaporate the ethanol.

Cap the tube and vortex the sample. If the sample is viscous, add 100 ul of ddH_2O or TE buffer pH 8.0 and incubate for 2 minutes. Continue this procedure until a smooth, syrup-like consistency is achieved.

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