ความสัมพันธ์ระหว่าง ยีน *HLA-E* กับความเสี่ยงทางพันธุกรรมของการเกิดโรคมะเร็งโพรงหลังจมูก

นางสาวอิงอร กิมกง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545

ISBN 974-17-1859-4

THE ASSOCIATION BETWEEN *HLA-E* GENE AND GENETIC SUSCEPTIBILITY OF NASOPHARYNGEAL CARCINOGENESIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Microbiology
Inter-Department of Medical microbiology
Graduate school
Chulalongkorn University
Academic Year 2002

ISBN 974-17-1859-4

Thesis Title	The Association Between *HLA-E* gene and Genetic Susceptibility of Nasopharyngeal Carcinogenesis
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Field of study	Medical Microbiology
Thesis Advisor	Assistant Professor Nattiya Hirankarn

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อิงอร กิมกง : ความสัมพันธ์ระหว่าง ยีน *HLA-E* กับความเสี่ยงทางพันธุกรรม ของการเกิดโรคมะเร็งโพรงหลังจมูก (THE ASSOCIATION BETWEEN *HLA-E* GENE AND GENETIC SUSCEPTIBILITY OF NASOPHARYNGEAL CARCINOGENESIS)
อาจารย์ที่ปรึกษา : ผศ.พญ.ดร. ณัฏฐิยา หิรัญกาญจน์, 96 หน้า. ISBN 974-17-1859-4

มะเร็งโพรงหลังจมูกเป็นมะเร็งที่มีลักษณะการเกิดโรคที่จำเพาะในบางภูมิภาค พบได้น้อยในประเทศทางตะวันตก แต่มีอุบัติการณ์สูงในประเทศจีนตอนใต้ และในประเทศแถบเอเชียตะวันออกเฉียงใต้รวมทั้งประเทศไทย ปัจจัยที่เป็นสาเหตุของการเกิดโรคมะเร็งโพรงหลังจมูกคือ ปัจจัยทางด้านสิ่งแวดล้อม และปัจจัยทางด้านพันธุกรรม คนจีนที่อพยพย้ายถิ่นฐานไปยังประเทศต่างๆ พบว่ามีอุบัติการณ์ของโรคนี้สูงกว่าชนพื้นเมืองในประเทศนั้น ๆ บ่งชี้ว่าปัจจัยทางด้านพันธุกรรมของตัวมนุษย์เองมีส่วนสำคัญในการเกิดโรคมะเร็งโพรงหลังจมูก การศึกษาก่อนหน้านี้รายงานความสัมพันธ์ระหว่างมะเร็งโพรงหลังจมูกกับ Human leukocyte antigen (HLA) เช่น HLA-A2 และHLA-B46 ซึ่งทำหน้าที่นำเสนอแอนติเจนให้แก่ T lymphocyte ซึ่งมีความสำคัญต่อการติดเชื้อไวรัส อย่างไรก็ตามในปัจจุบันนักวิทยาศาสตร์ยังไม่มีหลักฐานที่สามารถอธิบายความสัมพันธ์นี้ได้อย่างชัดเจน การศึกษาต่อมาช่วยสนับสนุนว่ายีนที่กำหนดความเสี่ยงของการเกิดโรคมะเร็งโพรงหลังจมูกไม่ใช่ยีน HLA-A และ HLA-B แต่น่าจะเป็นยีนอื่นที่อยู่ภายในบริเวณ HLA region ซึ่งอยู่ใกล้กับ D6S1624 microsatellite locus งานวิจัยนี้จึงสนใจศึกษายีน HLA-E ที่อยู่ใกล้กับ D6S1624 microsatellite locus และมีความสำคัญต่อหน้าที่ในการฆ่าของ NK cell และ CTL งานวิจัยนี้ทำการศึกษาความสัมพันธ์ระหว่างยีน HLA-E กับการเกิดโรคมะเร็งโพรงหลังจมูก โดยการวิเคราะห์ SNP ของยีน HLA-E ในผู้ป่วยโรคมะเร็งโพรงหลังจมูกเปรียบเทียบกับคนปกติในกลุ่มที่มีเชื้อสายเดียวกัน ซึ่งประกอบด้วย คนไทย คนจีน และคนไทย-จีน โดยใช้วิธี PCR-SSOP หารูปแบบของ SNP 7 ตำแหน่ง ในผู้ป่วย 174 คน และในคนปกติ 200 คน ผลการศึกษาพบว่ามี 2 ตำแหน่ง คือ codon 77 และ 107 ที่มีความแตกต่างในลำดับเบส ซึ่ง SNP ที่ codon 77 เป็นตำแหน่งที่ไม่เปลี่ยนกรดอะมิโน ในขณะที่ codon 107 มีการเปลี่ยนกรดอะมิโน จาก arginine เป็น glycine สำหรับการวิเคราะห์ SNP ที่ codon 107 พบว่า มีความสัมพันธ์กันระหว่าง HLA-E107G allele กับกลุ่มผู้ป่วยโรคมะเร็งโพรงหลังจมูกที่มีเชื้อสายไทย อย่างมีนัยสำคัญทางสถิติ (OR (95% CI)= 1.78 (1.16-2.74), p=0.006) ส่วนผลของ HLA-E107G allele จะมีลักษณะคล้ายการถ่ายทอดแบบยีนด้อย ซึ่งก็คือ ต้องการ 2 alleles ในการเพิ่มความเสี่ยง ซึ่งได้ค่าปัจจัยเสี่ยง คือ OR (95% CI) = 2.11 (1.15-3.88) และ p=0.009 นอกจากนี้ การวิเคราะห์ haplotype ของ SNPs ทั้งสองตำแหน่งได้ยืนยันบทบาทของ HLA-E107G ในการเกิดโรคมะเร็งโพรงหลังจมูก ผลสรุปคือ HLA-E107G allele อาจจะมีความสำคัญในการเกิดโรคมะเร็งโพรงหลังจมูกในคนไทย ซึ่งอาจจะเกี่ยวข้องกับการยับยั้งหน้าที่ของ NK cell หรือ CTL ที่จำเพาะต่อเซลล์มะเร็งที่ติดเชื้อ EBV

สหสาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....อิงอร กิมกง.....
สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2545

##4389121420 : MAJOR MEDICAL MICROBIOLOGY

KEYWORD : NASOPHARYNGEAL CARCINOMA / HLA-E / SNP / PCR-SSOP / HAPLOTYPE

INGORN KIMKONG : THE ASSOCIATION BETWEEN *HLA-E* GENE AND GENETIC SUSCEPTIBILITY OF NASOPHARYNGEAL CARCINOGENESIS. THESIS ADVISOR : ASSIST. PROF. NATTIYA HIRANKARN, M.D. Ph.D., 96 pp. ISBN 974-17-1859-4

Nasopharyngeal carcinoma (NPC) is a geographically restricted tumor. The tumor is rare in the western world but more prevalent in Southern China and many Asian countries including Thailand. The etiologic factors identified for NPC include environmental and genetic factors. Emigrant Chinese individuals in several countries showed a higher incidence of NPC than the indigenous individuals suggested that the genetic of host play a major role for NPC development. Previous studies reported the association between NPC and HLA (human leukocyte antigen) such as HLA-A2 and HLA-B46, which present antigen to T lymphocyte that is important in viral infection and tumor. However, the explanation of this association is still unclear. Latter studies suggested that the NPC susceptibility genes are not the HLA-A or HLA-B genes, but may be other genes within the HLA region, near the D6S1624 microsatellite locus. In our study, we are interested in HLA-E, which is in close proximity to D6S1624 microsatellite locus and plays important role in NK and CTL – mediated lysis. This study was conducted to compare SNP of the HLA-E gene between NPC patients and normal controls of the same ethnic origins; Thai, Chinese and Thai-Chinese. Seven positions of SNPs in HLA-E region were determined by PCR-SSOP in 174 patients with NPC and 200 healthy blood donors. As the results, only two SNPs (codon 77 and 107) show polymorphism. While changes in codon 77 is a silent mutation, codon 107 polymorphism leads to a missense mutation changing arginine to glycine. Analysis of SNP pattern at codon 107 shows a significant association between HLA-E107G allele and NPC patients of Thai origin with OR (95% CI), 1.78 (1.16-2.74), p=0.006. The effect of HLA-E107G was similar to that of an autosomal recessive gene, in which two homozygous alleles, but not heterozygous, were required to increase the relative risk, OR (95% CI), 2.11 (1.15-3.88), p=0.009. In addtion, haplotype analysis of two HLA-E SNPs, 77C/T and 107A/G has confirmed the role of HLA-E107G for NPC development. In conclusion, HLA-E107G allele might be important in the nasopharyngeal carcinogenesis of the Thai population, possibly via inhibition of NK cell or CTL function against EBV infected - tumor cell.

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Academic year 2002

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I want to thank the committee of Medical Microbiology Program for giving me permission to commence this thesis in the first instance, to do the necessary research work. I am deeply indebted to my advisor Assistant Professor Dr.Nattiya Hirankarn, for her stimulating guidance, valuable advice and supporting. Her expertise in immunology improved my research skills and prepared me for future challenges. I would like to thank Associate Professor Dr.Apiwat Mutirangura for his kindness and supporting in DNA samples.

I special thank to Mr. Wichai Pornthanakasem for helping in laboratories. I am so grateful to Miss Rungnapa Hirunsatit and Miss Narisorn Kongruttanachok for inspiration and my colleagues, Miss Thidathip Wongsurawat and Mr. Jeerawat Nakkuntod for their assistance. If I lacked them, this work would not be accomplished. Finally, I would like to express my deepest gratitude to my parents and sisters for their love, supporting, encouragement and understanding.

This work was supported by the Ministry of University Affairs (MUA)-CU Thesis Grant and Molecular Biology Project, Faculty of Medicine, Chulalongkorn University.

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ABBREVIATIONS

bp	base pair
CD	Cluster of Differentiation
95% CI	95% Confidence Interval
cM	Centimorgan
CTL	Cytotoxic T Lymphocyte
^{o}c	degree celsius
EBV	Epstein Barr Virus
EH	Estimating Haplotype
ER	Endoplasmic Reticulum
et al	et alii
kDa	Kilodalton
HLA	Human Leukocyte Antigen
µl	microliter
µg	microgram
ml	millliliter
mM	millimolar
ng	nanogram
NK	Natural Killer
NPC	Nasopharyngeal Carcinoma
OR	Odd Ratio
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl sulfate
SNP	Single Nucleotide Polymorphism
SRP	Signal Recognition Particle
SSC	Standard saline citrate
SSOP	Sequence Specific Oligonucleotide Probe
TCR	T Cell Receptor
U	Unit
vβ	variable beta

CHAPTER I

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a geographically restricted tumor of epithelial cell lining nasopharynx (Chan et al., 1985; Ren and Chan, 1996). The tumor is rare among European and North American Caucasians (less than 1 per 100,000) (Liebowitz, 1994). However, it is more common in many Asian countries, especially in Southern China (30 - 50 per 100,000) (Ren and Chan, 1996). The etiologic factors identified for NPC include environmental and genetic factors. Implicated environmental factors include the Epstein Barr virus, nutritional factors and exposure to smoke, wood dust and aromatic hydrocarbon (Lee et al., 1994; Zheng et al., 1994; Armstrong and Eng, 1983). The data of different incidence among different populations as well as the high incidence among migrants and family clustering suggested the involvement of genetic factors in addition to environmental factors (Chan et al., 1985).

The roles of susceptibility genes have been studied by using candidate gene approach, especially the genes that associated with Epstein Barr virus infection. The genes that have been associated with NPC patients include certain human leukocyte antigen (HLA) such as HLA-A and HLA-B (Ren and Chan, 1996). The physiological function of these genes is to present peptide to interact with T cell receptor on $CD8^+$ T cell, which is important in viral infection (Pamer and Cresswell, 1998). However, the explanations of these associations are still unclear. Another possibility is that there may be another disease susceptibility gene, which linked to the HLA-A, and HLA-B genes. This latter hypothesis was further strengthened by Lu and coworker in 1990 who conducted a linkage study based on affected sib-pair in Chinese population which suggests that, a gene closely linked to the HLA locus confers a greatly increased risk of nasopharyngeal carcinoma (Lu et al.,1990). Since the suspected gene is around the HLA region, Ooi and co-workers began mapping the approximate position of a gene closely linked to the HLA region by screening the NPC samples with highly polymorphic

microsatellite markers and HLA genes that are scattered throughout the human HLA, and compared them with normal healthy controls. In that study, they found that only D6S1624 showed alleles with significant difference in frequencies between NPC and controls, which showed a relative risk at approximately 3.5. Thus, NPC susceptibility gene may be within the centromeric end of the class I and the telomeric end of class III regions of HLA, near the D6S1624 microsatellite locus (Ooi, Ren and Chan, 1997).

In our study, we are interested in the HLA-E gene that is in close proximity to this microsatellite marker. HLA-E belongs to a non-classical HLA class Ib group of molecules which are homologous to classical HLA class Ia molecules but are characterized by a limited polymorphism and low cell surface expression (Shawar et al., 1994). HLA-E is transcribed in most cells and tissues (Wei and Orr, 1990). The role of HLA-E is to interact with NK cell receptor (Lazetic et al., 1996). Specific function of NK cell is to kill viral infected and tumor cells. HLA-E involves in the regulation of natural killer cell function by presenting leader peptide derived from HLA-A, B, C and G molecules. Binding of these leader peptide to HLA-E stabilizes HLA-E, which then migrate to the cell surface and interact with CD94/NKG2 receptor on NK cells (O'Callaghan, 2000). The dominant interaction of HLA-E with NK cells appear to result in inhibition of NK cell – dependent lysis, mediated by the inhibitory CD94/NKG2A receptor (Houchins et al., 1997). However, CD94/NKG2C receptors that activate NK cells, can also interact with HLA-E (Lanier, 1998). The functional significance of the interaction of HLA-E with is activatory receptor is currently unclear. Recent study has shown that different HLA-E alleles can affect different functions of natural killer cells (Maier et al., 2000). Those results indicate that single amino acid substitution is important to NK cell functions. One study showed a significant decrease in NK cell activities of NPC patients compared to normal controls (Lynn et al., 1986). We hypothesized that polymorphic patterns of the HLA-E gene in NPC patients may differ from normal controls resulting in the decrease of NK cell activities. Several observations raise the possibility that HLA-E might play an important role in the regulation of CTL function, as CD94/NKG2 receptors are also expressed on a subset of CTL cells (Mingari, Moretta and Moretta, 1998; Carena et al., 1997; Le Drean et al., 1998). Furthermore, there is evidence that HLA-E

can interact with T cell receptors (TCRs). The data imply the generation of human T cells potentially recognizing through the αβTCR-HLA-E molecules that bind to class I- and virus-derived peptides (Garcia et al., 2002). One indirect evidence included the observation that Qa-1b, a mouse homologue of HLA-E has been proposed to present H-2-unrelated antigenic peptides that may be specifically recognized by T lymphocytes (Bouwer et al., 1998; Jiang et al, 1998; Lowen et al, 1993; Lo et al., 2000; Jiang and Chess, 2000). Thus, in this study, single nucleotide polymorphisms (SNPs) within the HLA-E gene used as polymorphic markers were analyzed based on positional-candidate gene approach. SNPs analysis was determined by the polymerase chain reaction (PCR), with primers specific for exon 2 and exon 3 of the HLA-E gene, in combination with dot-blot hybridization with 12 sequence specific oligonucleotide probes (SSOPs). Then analysis of SNP patterns between NPC patients compare to control group was done by Chi-square.

We hypothesized that the HLA-E SNP patterns specific for NPC patients might suggest the role of HLA-E as an NPC susceptibility gene and might lead to a better understanding of the NPC carcinogenesis. This finding might be applied to detect early stage NPC patients or patients at high risk of NPC. In addition, our study will provide of of the basic knowledge of the diversity of HLA-E in Thai population.

CHAPTER II

OBJECTIVE

The objective of this study was:

To study the association between the HLA-E gene and nasopharyngeal carcinogenesis by SNPs analysis of the HLA-E gene in NPC patients and control group using PCR-SSOPs (polymerase chain reaction-sequence specific oligonucleotide probes).

CHAPTER III

LITERATURE REVIEW

Nasopharyngeal carcinoma (NPC)

Classification

Nasopharyngeal carcinoma (NPC) is a cancer of epithelial cell lining nasopharynx (Ren and Chan, 1996). The world Health Organization (WHO) has developed a classification system that divides nasopharyngeal carcinomas into three types based on light microscopic finding (Shanmugaratnam et al., 1993)

1. WHO type 1 or keratinizing squamous cell carcinomas

Keratinizing squamous cell carcinomas are characterized by moderate to well differentiated cells that produce keratin. Twenty-five percent of NPC are of this type.

2. WHO type 2 or Non- keratinizing squamous cell carcinomas

Non- keratinizing squamous cell carcinomas have cells that vary from mature to anaplastic in appearance but produce minimal if any keratin. Approximately fifteen percent of NPC are of this type.

3. WHO type 3 or undifferentiated NPC

This type comprises a diverse group of carcinomas often described as undifferentiated carcinomas. Sixty percent of all NPC are of this type. Undifferentiated NPC are the most common and are associated with the Epstein Barr virus (Morgan, Murray and Challiss, 1992)

Epidemiology

Nasopharyngeal carcinoma (NPC) is a cancer with a particular geographic distribution. In general, this cancer is rare in the world with incidence rate of half to two cases per 100,000 population per year (Fandi et al., 1994). NPC accounts for fewer than one percent of cancers in North American and Western Europe with incidence rate of less than one per 100,000 population per year (Liebowitz, 1994). However, this cancer has an intermediate incidence of 3 to 10 cases per 100,000 population per year in inhabitants of Northern China, the Mediterranean basin, Northern Africa, and Southeast Asia. Among the Inuit in Alaska and Greenland, the incidence of NPC is increased to 15 to 20 cases per 100,000 population per year (Fandi et al., 1994). In southern China, NPC has a much higher incidence, with documented rates of 30-50 cases per 100,000 population per year (Ren and Chan, 1996). NPC is ranked as the tenth most common cancer in Thailand, with incidence rate of 3.6 cases per 100,000 population per year (National Cancer institute of Thailand [NCI], 1993). This cancer may occur at any age, but the peak incidence is in persons 40 to 50 years of age. Males outnumber females by approximately 3.5: 1 (Fandi et al., 1994).

Etiology

Many factors may influence the development of NPC including environmental factors and genetic susceptibility.

1. Environmental factors

Implicated environmental factors include nutritional factors, exposure to smoke, wood dust and the Epstein Barr virus.

The consumption of salted fish and other salt preserved foods, including eggs, leafy vegetables and roots, in early childhood has been documented as a substantial

risk factor for development of NPC in Malaysian Chinese (Armstrong et al., 1998) Similarly, salted fish consumption in early childhood has been correlated with an unusually high incidence of NPC in the boat communities of Hong Kong's harbors. N-nitrosodimethylamine in salted fish, perhaps in combination with vitamin deficiency, has been considered as a likely carcinogen (Chan et al., 1985; Fandi et al., 1994).

Occupational hazards, including exposures to formaldehyde, dust and smoke particulate and certain aromatic hydrocarbons, have been investigated as risk factors for NPC (Farrow et al., 1998; Vaughan et al., 2000, Mirabelli et al., 2000).

Epstein Barr virus (EBV), a herpesvirus, is a causative agent in acute infectious mononucleosis and is also associated with Hodgkin' s disease, Burkitt' s lymphoma, lymphoproliferative disease in the post-transplant setting, and T cell lymphoma (Liebowitz and Kieff , 1993). EBV initiates an early active (or lytic) infection and persists in a latent stage until it is reactivated under certain conditions of immunosuppression or illness. The link between NPC and EBV was first observed in 1966, when the sera of patients with the malignancy were found to manifest precipitating antibodies against cells infected with the virus (Old and Boyse, 1966.). The association between EBV and NPC was subsequently confirmed by EBV nucleic acid hybridization studies on NPC biopsy material and by demonstrating that EBV DNA was present in the NPC tumor cells. Furthermore, one experiment has shown that EBV is capable of causing malignant change in cell culture and suggests a role for the virus in contributing to the transformation event (Liebowitz, 1994).

2. Genetic susceptibility

Genetic susceptibility has also been proposed as a risk factor for the development of NPC. Many of the migrant studies were reported to support the hypothesis of genetic risk factors. For example, higher trend of the risk for NPC development was shown in emigrant Chinese to California, Singapore and Taiwan (Clifford, 1970; Simons et al., 1976; Chan et al., 1983; Lin et al., 1986). In addition, there

were numerous reports of familial nasopharyngeal cancer (Fisher, Fisher and Cooper, 1984; Ko et al., 1998).

Gene identification

In principle, there are four main ways to identify and isolated genes responsible to genetic disorders.

1. Functional cloning approach

In this approach one has to have some prior knowledge of the detailed gene functions associated with the pathogenesis. The classical example is Sickle Cell Anemia (SCA). Even before the advent of gene cloning it was known that this disease was caused by a mutation in one of the two genes which encode either alpha or beta – globin which come together to form Hemoglobin. The cloning of the beta-globin gene indeed confirmed that it was mainly mutations in this gene which gave rise to SCA or even thalassemias. Although the first prove came back in 1950s by Vernon via peptide sequencing. In principle, the Functional cloning approach is a powerful technique but sadly impossible with most genetic disorders with no prior information of the proteins involved.

2. Positional cloning approach

Positional cloning approach does not require any prior knowledge of the proteins involved. This approach rests on linkage analysis using extended families containing individuals with a heritable condition. Genetic and physical maps lead to identification of disease genes.

3. Candidate gene approach

Rather than searching for a relative gene randomly throughout the genome, it is desirable to focus on genes that may already be suspected to have a role in the pathophysiology of the disease. This approach has been widely used for the association study of complex diseases (Tabor, Risch and Myers, 2002).

4. Positional - Candidate gene approach

This strategy using a combination of positional and candidate gene approaches. It is powerful to identify a disease gene in the present day.

Polymorphic markers

The technique of positional cloning as a general strategy for the isolation of human disease genes is based upon the fact that any detectable differences in DNA sequences between individuals can be used as genetic markers in human DNA. The linkage analysis can exploit these markers as references to determine and map the position of other genes. There are several types of markers that available in recent day.

1. Restriction fragment length polymorphism (RFLP)

RFLPs were the first generation marker that based on the inherited differences in cleavage sites for restriction endonucleases (Strachan and Read, 1999). A single base change within a restriction endonuclease site is a readily detectable marker since the enzyme in question can no longer cleave the mutation site. This technique allowed the identification of only two alleles per locus. Modifications of the RFLPs technique are possible to identify multiple alleles, but still have practical drawbacks that make preferable the use of the PCR based microsatellite markers.

2. Minisatellites

Minisatellite VNTR (variable number tandem repeat) markers were a great improvement. The VNTRs have many alleles and a high rate of heterozygosity. Thus, most meioses are informative. However, the technical problems of Southern blotting and radioactive probes were still an obstacle to easy mapping, and VNTRs are not evenly spread across the genome (Strachan and Read, 1999).

3. Microsatellites

Microsatellites systems are composed of tandem repeats of short nucleotide sequences. They are distributed throughout the genome in a much higher frequency than RFLPs. Microsatellite facilitated the construction of genome maps in most livestock species because their abundance in the genome, the specificity of the primers, a high degree of polymorphism and their easy detection.

4. Single nucleotide polymorphism (SNP)

SNPs are the most common form of DNA sequence variations that occur when a single nucleotide in the genome sequence is changed. They are useful polymorphic markers to investigate genes susceptible to diseases or those related to drug responsiveness. A map of human genome sequence variations containing 1.42 million SNPs, provids an average density on available sequence of one SNP every 1.9 kilobases. In contrast to more mutable markers, such as microsatellites, SNPs have a low rate of recurrent mutation, making them stable indicators of human history (Sachidanandam et al., 2001)

The roles of susceptibility genes for NPC

The roles of susceptibility genes for NPC have been studies by using candidate gene approach; especially the genes that associated with EBV infection, which can divided into 3 types.

1. The genes associated with entering of the EBV into epithelial cells such as complement receptor 2 (CR2) and polymeric immunoglobulin receptor (pIgR) (Lin et al.,1997; Mostov, 1994; Zhang et al., 2000; Hirunsatit et al., 2003).

2. The gene which interact with EBV protein and have important role in cell cycle and apoptosis such as TNF, TNFR, p53 and Rb. (Golovleva et al., 1997; Leung et al., 1998; Feng et al., 1999; Kuo et al., 1994).

3. The genes involved in the immune system such as HLA-A, HLA-B, IL-10, IFNA and TCR (Ren and Chan, 1996; Pimtanothai et al., 2002; Bejarano and Masucci,1998; Golovleva et al., 1997; Chen and Chan,1994; Silins et al., 1998).

The association of NPC with HLA

An association between the HLA profile and risk for nasopharyngeal carcinoma (NPC) among Chinese in Singapore was first reported in 1974 (Simons et al., 1974). Increased incidence of disease was associated with the antigen A2 and with a lack of detectable antigens at locus B. The major part of the locus B blank was later identified as a new antigen, BW 46 (originally Sin 2), and this antigen was shown to be associated with high risk for NPC, with a relative risk of about 2 (Simons et al., 1975). In subsequent reports by Chan SH in 1983, the study showed that the association among Chinese NPC were with A2 BW46 among older onset NPC and AW19 B17 among total NPC but this association was especially strong among younger onset NPC (Chan et al., 1983). The

association of A2 and BW46 with NPC was observed in a recent study among Southern Chinese (Goldsmith, West and Morton, 2002). The data of Zhang (1981) showed that HLA-A1 and HLA-BW63 were associated with NPC in Hunan or Southern China (Zhang, 1981). Another HLA antigen which is associated with NPC is B58, which occurs in both Chinese and Malay patients with NPC (Ren and Chan, 1996). Daniilidis et al (1997) reported a significant associated between the haplotype HLA-B5 DR11 and NPC in 53 Greece patients (Daniilidis et al., 1997). Among Scandinavian-American families, a specific HLA haplotype of A1-B37-DR6 was associated with a predisposition for NPC, but no linkage was identified (Coffin, Rich and Dehner, 1991). Among North African NPC, the frequencies of A3, B5 and B15 were increased and AW33, B14 and DR4 decreased (Herait et al., 1983) and yet in another North African population, the Tunisians, no HLA association was found (Betuel et al., 1975). Recently, using serologic and molecular methods, 45 NPC patients were typed for HLA class I and class II and were compared to 100 unrelated normal Tunisian. The results showed that the antigen frequency of HLA-B13 and allelic frequencies of DRB1*03, DRB1*15 were significantly higher in the NPC patients than in the control group (15.5 vs. 4; 26.4 vs. 11.5, and 14.4 vs. 6.5%, respectively) probably indicating a positive association with NPC (Mokni-Baizig et al., 2001). The data of Moore et al (1983) showed that twenty-four Caucasoids with NPC were HLA typed (24 AB, 16 DR). No associations were found in comparisons with normal healthy controls (Moore et al., 1983). Furthermore, many studies were reported that no significant HLA-C were found to be associated with Chinese and Caucasian populations (Zhu et al.,1990; Wu et al., 1989; Burt et al., 1996). In Thailand, the associations among Thai NPC were with HLA-B46 and HLA-B*51012 (Pimtanothai et al., 2002).

The physiological function of these HLA genes is to present peptide to interact with T cell receptor on $CD8^+$ T cell, which is important in viral infection (Pamer and Cresswell, 1998). However, the explanations of these associations in NPC are still unclear. It is also possible that there may be another disease susceptibility gene linked to the HLA-A and HLA-B genes confers a greatly increased risk of NPC (Lu et al., 1990). One study has reported that NPC susceptibility gene may be within the centromeric end

of the class I and telomeric end of the class III region of HLA, near the D6S1624 microsatellite locus which lies between HLA-A and HLA-B (Figure 1) (Ooi et al., 1997). However, later the exact localization of this microsatellite is proved to be on chromosome 6 at 6p and genetic distance approximately 44.9 centimorgan (cM; 1 cM=1,000 kb) (Foissac and Cambon-Thomsen, 1998), which is indeed very closed to the HLA-A locus. The localization of D6S1624 microsatellite locus was shown in Figure 2.

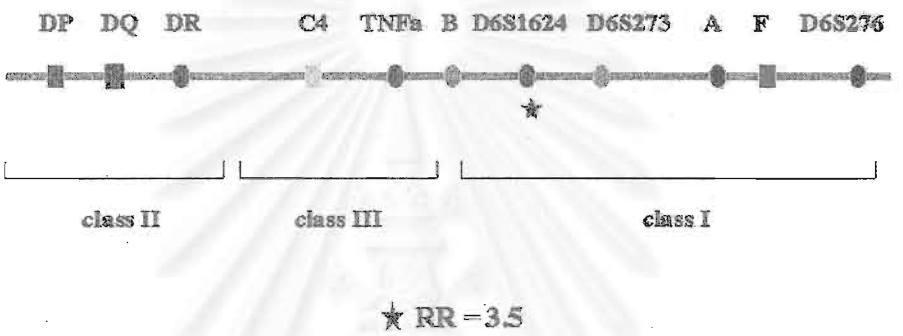


Figure 1 The position of D6S1624 microsatellite locus (Ooi et al., 1997).

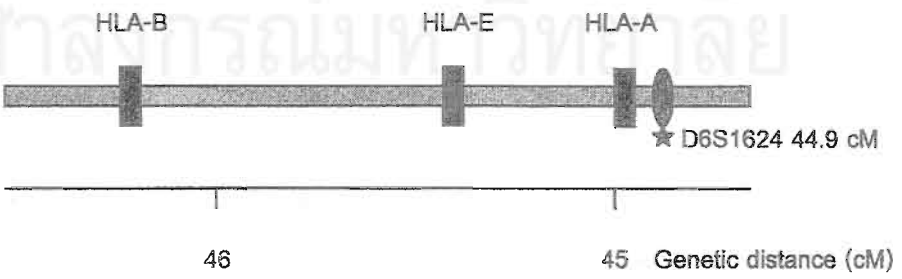


Figure 2 The localization of D6S1624 microsatellite locus (Foissac and Cambon-Thomsen, 1998).

Human leukocyte antigen (HLA)

The extended collection of genes on the short arm of human chromosome 6 at 6p21.3 is called the human leukocyte antigen (HLA). The locus was originally identified and named because of its role in tissue rejection following transplantation. Many genes (>10%) have been shown to have important functions in the biology of the immune system. Antibody (B cell humoral) and T cell mediated immune responses are initiated through genes contained within the HLA complex, so in this sense the HLA represents the front end of the adaptive immune response to invading pathogens. A large amount of scientific effort has gone into the understanding of this gene complex by studying the genes encoded within the region and the role of the complex in immunology and human pathology, including diseases of both infectious and genetic origin (Klein, 1986; Urban, 1996).

The genetics of the HLA

The HLA complex is a gene cluster of various loci grouped together on single region. Gene mapping studies have indicated that the HLA complex occupies 3.5 megabases of DNA on the short arm of chromosome 6 in the distal portion of the 6p21.3 band. The loci of the HLA complex may be divided into three classes: class I, class II and class III (Roitt, Brostoff and Male, 1996). The organization of the genes that encode HLA molecules is shown in Figure 3.

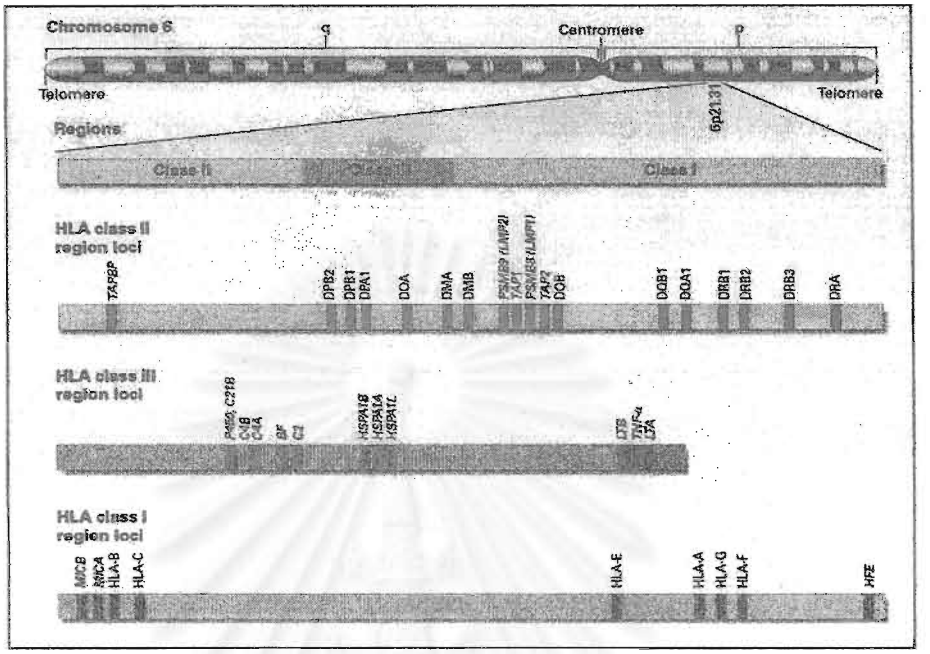


Figure 3 Organization of the HLA complex on chromosome 6 (Klein and Sato, 2000).

1. Class I HLA genes

The class I genes of the HLA complex can be divided into 2 types. There are classical HLA class Ia and non-classical HLA class Ib. The classical HLA class Ia contains three loci, called HLA-A, HLA-B and HLA-C, spreading over a region of 2 Mb(Guillaudeux et al.,1998). The HLA-E, -F, –G, HFE (HLA-H), MICA and MICB genes encode non-classical HLA class Ib proteins (Pook et al., 1991; Braud, Allan and McMichael, 1999). The class I HLA genes appear to be coordinately controlled with the beta two-microglobulin gene located on chromosome two. Although the beta two-microglobulin gene is not an HLA gene, the gene does code for a subunit associated with the heavy chain of HLA class I molecule. Studies indicate that the expression of the class I molecule is dependent upon the expression of the beta two-microglobulin gene (Jordan et al., 1985). A class I gene consist of eight exons. The first exon encodes a signal peptide that directs the insertion of the HLA class I molecule into the endoplasmic

reticulum during translation. Exon two through four encodes the three external domains of the protein (alpha 1-3). The fifth exon encodes the transmembrane domain, while exons six through eight encode cytoplasmic domains. Polymorphisms are on exons two and three. A comparison of the exons of a class I gene with domains of a class I polypeptide is shown in Figure 4.

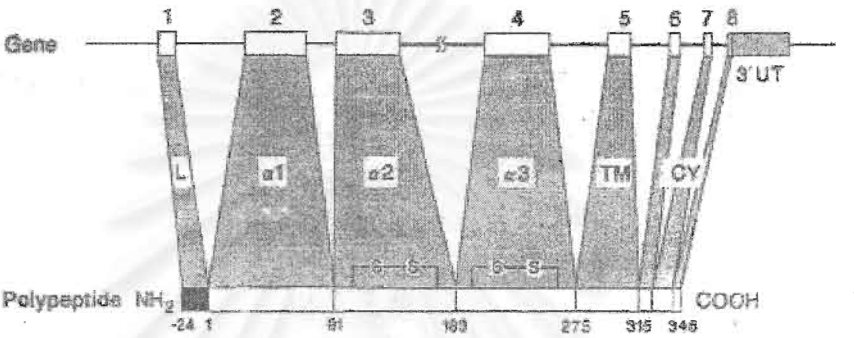


Figure 4 Gene and protein structure of class I (William, 1989).

2. Class II HLA genes

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

region in the alpha genes and the rest of the cytoplasmic domain of the beta genes (William, 1989).

3. Class III HLA genes

The class III HLA genes encode complement components (C2, C4 and factor B) that show no structural similarity to either class I or class II molecules (Roitt et al., 1996). These genes, along with genes encoding heat-shock protein-70 components and the peptide transporters that function in the loading of HLA class I molecules.

HLA polymorphism

One of most striking features of the HLA is the extensive polymorphism of the class I and II genes and their encoded products. This includes both isotypic and allelic variations on the basic antigen structures. The haploid copy number for genes of a given type will vary considerably depending on the species and on the genetic background. The number of definable allelic products of a given locus in a species has been increased by development of new methodology. The HLA Sequence Database which holds information on HLA sequences currently contains 1,620 allele sequences. In addition to the physical sequences, the database contains detailed information concerning the material from which the sequence was derived and data on the validation of the sequences. To date (January 2003), some 266 HLA-A, 511 HLA-B, 128 HLA-C, 6 HLA-E, 1 HLA-F and 15 HLA-G class I alleles have been named. A total of 3 HLA-DRA, 403 HLA-DRB, 23 HLA-DQA1, 53 HLA-DQB1, 20 HLA-DPA1, 101 HLA-DPB1, 4 HLA-DMA, 6 HLA-DMB, 8 HLA-DOA and 8 HLA-DOB class II sequences have also been assigned. In addition, there are also 6 TAP1, 4 TAP2 and 54 MICA sequences (Marsh, 2003).

Structure of HLA molecules

There are two main kinds of HLA molecules, class I and class II. Classical HLA class I molecules are found on all nucleated cells, while class II molecules show a more restricted expression pattern. Class II molecules are always expressed by B-lymphocytes, interdigitating dendritic cells, and thymic epithelial cell. Other cells, such as macrophages, activated T lymphocytes and endothelial cells can be induced to express class II molecules by cytokine such as IFN-γ (William, 1989). Both kinds of molecules belong to a group of molecules known as the immunoglobulin supergene family, which includes immunoglobulins, T cell receptors, CD4, CD8 and others. The class I and class II structure are described in this topic.

1. Class I HLA molecules

Class I HLA molecules are composed of a heavy chain non-covalently associated with beta2-microglobulin. The HLA-encoded polypeptide is about 350 amino acids long and glycosylated, giving a total molecular weight of about 45 kDa. This polypeptide folds into three separate domains called alpha-1, alpha-2 and alpha-3. Beta two microglobulin is a 12 kDa polypeptide that is non-covalently associated with the alpha –3 domain. The structures of class I HLA molecule is shown in Figure 5 (Bower, 2002). Between the alpha-1 and alpha-2 domains lies a region bounded by eight antiparallel beta-pleated sheets on the bottom and two alpha helices on the sides. This region is capable of binding (via non-covalent interactions) a small peptide of eight to ten amino acids (Roitt et al., 1996; Janeway and Travers, 1996). The view of the peptide-binding region of a class I HLA molecule is shown in Figure 6 (Klein and Sato, 2000).

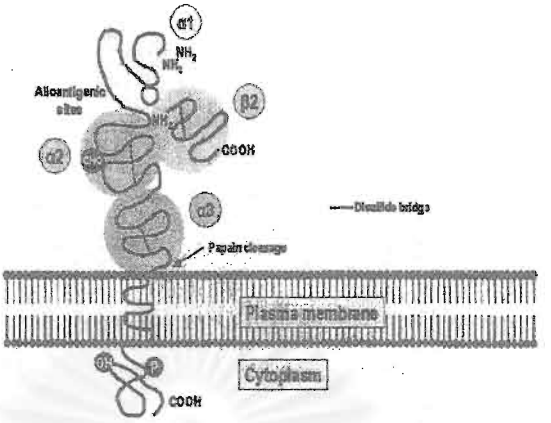


Figure 5 The structures of class I HLA molecule (Bower, 2002).

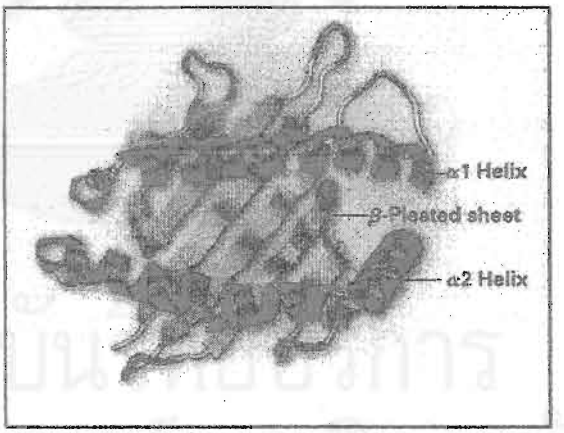


Figure 6 The view of the peptide-binding region of class I HLA molecule (Klein and Sato, 2000).

2. Class II HLA molecules

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

Molecular basis and functional of cell surface expression of HLA-E

1. Non–Classical HLA Class Ib

Non-classical HLA class Ib genes identified by molecular mapping, also are present in both the mouse and human HLA. In mice, the non-classical HLA class Ib genes are located in three region (H-2Q, T and M) downstream from the H-2 complex (M is not shown in Figure 7). In human, non-classical HLA class Ib genes include the HLA-E, -F, -G, -H, -J and –X loci as well as a recently discovered family of genes called MIC, which includes MICA through MICF (Goldsby et al., 2003) (Figure 7). HLA-E, -F and –G molecules have only limited polymorphism and are highly conserved (Sharwar et al., 1994; Boyson et al., 1995). Little is known about HLA-F, but HLA-G is expressed in the placenta and may play a role in reproductive immunology (O'Callaghan and Bell, 1998). HFE (formerly designated HLA-H) plays a role in iron metabolism. The MIC family has only 18%-30% sequence homology to classical HLA class Ia molecules, and those designated as MICA are highly polymorphic (Braud et al., 1999). In this study, we

are interested in the HLA-E gene. This gene is transcribed in most tissues, but with lower cell surface expression (Wei and Orr, 1990; Sharwar et al., 1994).

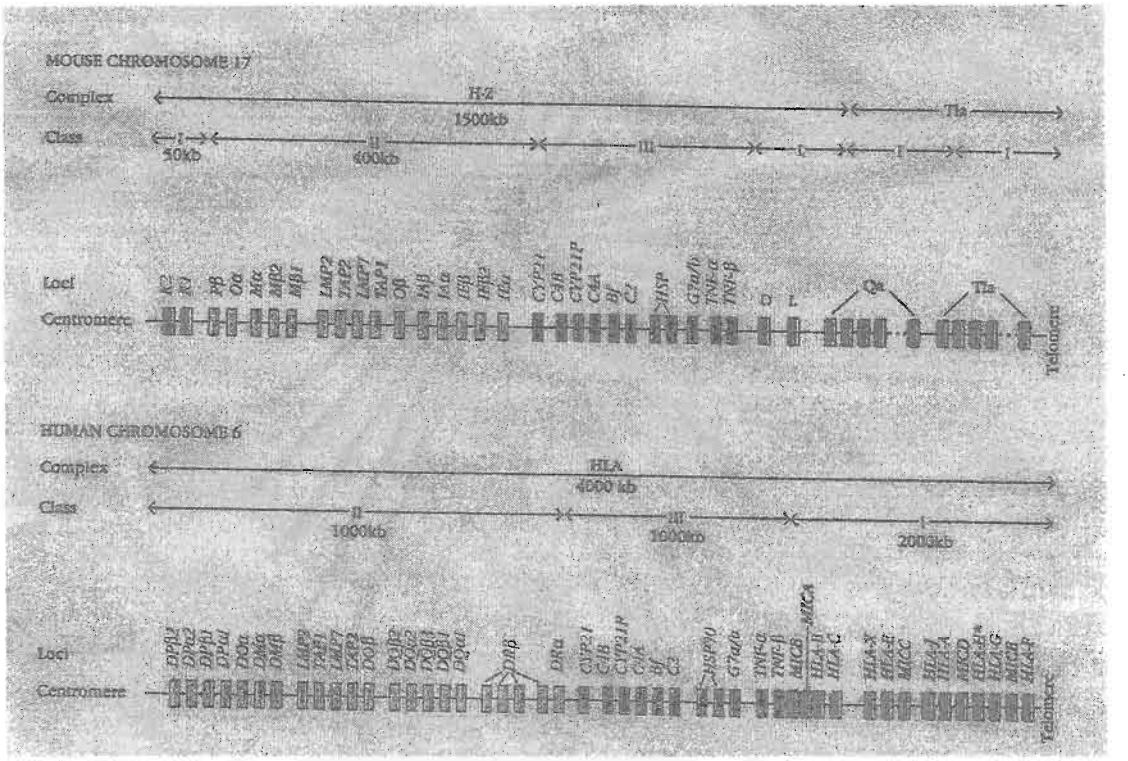


Figure 7 Map of the H-2 and HLA complexes (Goldsby et al., 2003).

2. The genetics of HLA-E

The HLA-E gene is similar to the other HLA class I genes that comprise eight exons. The first exon encodes a signal peptide that directs the insertion of HLA-E molecule into the endoplasmic reticulum during translation. Exons two through four encode the three external domains of the protein (alpha1-3). The fifth exon encodes the transmembrane domain, while exons six through eight encode cytoplasmic domains. The alpha-1 and –2 domains form a peptide binding region. This region is capable of

binding the leader peptide derived from the classical HLA-A, -B, -C and non-classical HLA-G (Braud, Jones and McMichael, 1997). The gene and protein structure of HLA-E are shown in Figure 4.

3. Structure of the HLA-E molecule

Structure studies of HLA-E have been revealed from the crystals of HLA-E in complex with Beta2-microglobulin and the HLA-B8 leader peptide (Figure 8) (O'Callaghan and Tormo et al., 1998). Overall, the structure framework of HLA-E is similar to that of the other human class I molecules that have been solved previously, such as HLA-A2 (O'Callaghan and Bell, 1998). The peptide is bound in a groove with sides formed by two alpha helices and a floor formed by a beta-pleated sheet (Figure 8). Beneath this beta-sheet, the alpha-3 domain and the interaction with beta2-microglobulin are conserved. However, the peptide-binding groove is highly specialized to provide an exquisitely specific binding site for the class I leader peptide (HLA-A, -B, -C and G) (O'Callaghan, 2000).

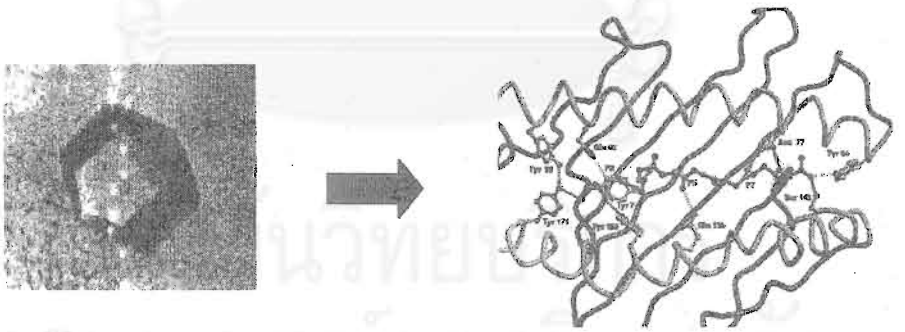


Figure 8 Crystallization of HLA-E and the crystallographic structure of the HLA-E groove (O'Callaghan, 2000; O'Callaghan and Tormo et al., 1998).

4. Cell biology of HLA-E

Significant insights into the biology of HLA-E were gained from studies on the mouse homologue Qa-1b. The mouse HLA class Ib molecule Qa-1 shares some characteristics with HLA-E in that it displays a broad tissue distribution, a very low level of cell surface expression, and some structural similarities in the peptide binding groove (Soloski et al., 1995). Although HLA-E differs from Qa-1b in 55 of 181 residues in the alpha-1 and –2 domains, it can bind leader peptide from human class Ia molecules which are very similar to the murine class Ia leader peptide bound by Qa-1b (Braud et al., 1997). HLA-E and Qa-1b, unlike other class Ia molecules, have serines rather than the conserved residues threonine and tryptophan at position 143 and 147 in the "F" pocket, respectively. In the "B" pocket, HLA-E and Qa-1b also share the key residues methionine and alanine at position 45 and 67, respectively (O'Callaghan and Bell, 1998). This shared and atypical amino acid usage suggested that HLA-E and Qa-1b might have similar functions (O'Callaghan, 2000). In addition, observations by Geraghty's group confirmed that HLA-E behaved in similar manner to Qa -1b (Lee and Goodlett et al., 1998). In a series of elegant experiments in mice, Qa-1b has been shown to bind to the conserved class I leader peptides derived from several mammalian species (Kurepa, Hasemann and Forman, 1998). The leader or signal peptide span about 20 amino acids, including a stretch of hydrophobic residues and is the first part of the protein to be synthesized by the ribosome. The leader peptides of these HLA proteins are highly conserved, despite the extensive polymorphism of the mature proteins (Figure 9). The leader peptide are recognized and bound by a signal recognition particle (SRP), which brings it and ribosome to endoplasmic reticulum (ER) by binding to the SRP receptor on the ER membrane. Binding to the receptor releases the SRP from both the ribosome and the leader peptide of the nascent polypeptide chain. The ribosome then binds to a protein translocation complex in ER membrane, and leader peptide is inserted into a membrane channel. Translocation is then able to resume, and the growing polypeptide chain is translocated across the membrane into the ER. As translocation proceeds, the leader peptide is cleaved by signal peptidase and the polypeptide is released into the lumen of ER (Thorpe, 1984) (Figure 10). Moreover, the finding of

Geraghty's group suggested that an HLA class Ia leader must be present in the cells for stable mature HLA-E protein to form and migrate to the cell surface (Lee and Goodlett et al., 1998). Another studies showed that HLA-E molecules preferentially bind peptides derived from the signal peptides of other HLA class I molecules by a TAP-dependent mechanism and do not bind HLA-E leader peptide itself (Braud et al., 1997; Lee and Goodlett et al., 1998; Braud et al., 1998).

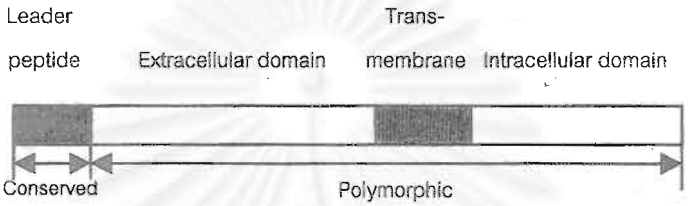


Figure 9 The arrangement of the newly synthesized classical HLA class Ia proteins (O'Callaghan, 2000).

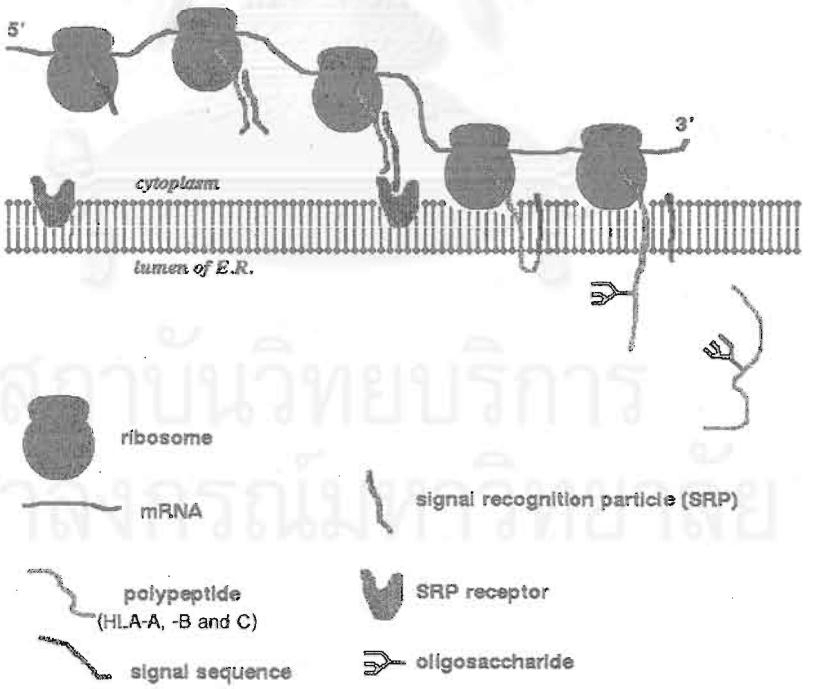


Figure 10 Synthesis of class Ia molecules (Walshaw, 1996).

5. Class Ia leader peptides

The class Ia leader peptides are known as HLA-A, -B and C. A standard leader sequence is conserved throughout the class I molecules, and is of the form MAVMAPRTVLLLSGALALTQTWA. Using synthetic peptides, it has been shown that the HLA-B8 leader peptide (VMAPRTVLL) can bind to HLA-E both *in vitro* in refolding assays and in cells using a modified assembly assay (O'Callaghan and Tormo, 1998; Braud et al., 1997). Several studies have also suggested that HLA-A2, -B7, -B15, -Cw1, -Cw3, -Cw*0401, -Cw*0402, -Cw7 and –Cw*1502 leader peptides are able to bind and stabilize HLA-E for cell surface expression (Lee and Goodlett et al., 1998; Braud et al., 1997; Stevens et al., 2001; Borrego et al., 1998; Maier et al., 2000). The leader sequence of HLA-G, which is non-classical HLA class Ib, also contains a peptide that is capable of binding HLA-E (VMAPRTLFL) (Braud et al., 1997; Lee and Goodlett et al., 1998; Braud et al., 1998; Maier et al., 2000). However, both HLA-E and F have shorter leader sequences (MVDGTLLL and MAPRSLLL respectively) which do not bind HLA-E (Braud et al., 1997). In addition, recent studies have shown that human CMV open reading frame UL40 encodes a canonical ligand for HLA-E, identical with HLA-Cw*0304 signal sequence-derived peptide. The data also indicated that HCMV gpUL40 can be processed and loaded onto HLA-E in the absence of a functional TAP complex, allowing an HLA-E-mediated inhibition of NK activity (Ulbrecht et al., 2000).

6. NK cells (Natural Killer cells)

NK cells are bone marrow-derived lymphocytes. Like cytotoxic T cells, they contain granules filled with potent chemicals such as perforin and granzyme. They form a distinct group of lymphocytes with no immunological memory and are independent of the adaptive immune system. Their specific function is to kill pathogen-infected or tumor cells and secretes soluble inflammatory mediators very early in infection. The " natural killer" activity of NK cells is regulated through two families of membrane receptors (O'Callaghan, 2000; Moretta et al., 2002).

7. NK cell receptors

Two major families of NK cell receptors have been identified in humans. Firstly, the KIR gene family comprises a multi-gene family of about 16 genes, whose products recognize polymorphic determinants on HLA-A, B and –C molecules (Colonna and Samaridis, 1995). The second family of receptors is C-type lectin superfamily, which is a heterodimers of CD94 disulphide-bonded to NKG2 (Lazetic et al., 1996). These C-lectin molecules are all encoded on chromosome 12p12.3-p13.1, a region syntenic to that of mouse chromosome 6 encoding the Ly49 genes. The function of CD94 is to allow transport of NKG2 proteins to the cell surface. NKG2 displays seven different isoforms comprosed of NKG2A, B, C, D, E, F and H (Brooks et al., 1997; Lazetic et al., 1996; Carretero et al., 1997; Bauer et al., 1999; Glienke et al., 1998; Bellon et al., 1999). These display either inhibitory (NKG2A and B) or activating functions (NKG2C, E, and H) (Brooks et al., 1997; Houchins et al., 1997; Carretero et al., 1997; Bellon et al., 1999; Bauer et al., 1999). The function of NKG2F is still not clear. The only known ligand for receptors composed of CD94 and either NKG2A, B or C is the HLA class Ib molecule HLA-E (Braud et al., 1998; Brooks et al., 1999).

8. The role of HLA-E in NK cell function

HLA-E binds to the leader peptide derived from the polymorphic classical HLA-A, HLA-B, HLA-C and non-classical HLA-G. This peptide binding is highly specific and stabilizes the HLA-E protein, allowing it to migrate to the cell surface. A functioning TAP (transporter associated with antigen processing) molecule is required to transport these peptides into the endoplasmic reticulum, where they can interact with HLA-E. HLA-E then migrates to the cell surface, where it interacts with NK cell receptors (O ' Callaghan, 2000). The dominant interaction of HLA-E with NK cells appear to result in inhibition of NK cell dependent lysis, mediated by the inhibitory CD94/NKG2A receptors. However, CD94/NKG2C receptors that activate NK cells, can also interact with HLA-E (Houchins et al., 1997). The functional significance of the interaction of HLA-E with these activatory receptors is currently unclear. Furthermore, recent study shows that different

HLA-E alleles can affect different inhibitory function of NK cell – mediated lysis (Maier et al., 2000). The proposed mechanism of HLA-E in NK cell function is shown in Figure 11.

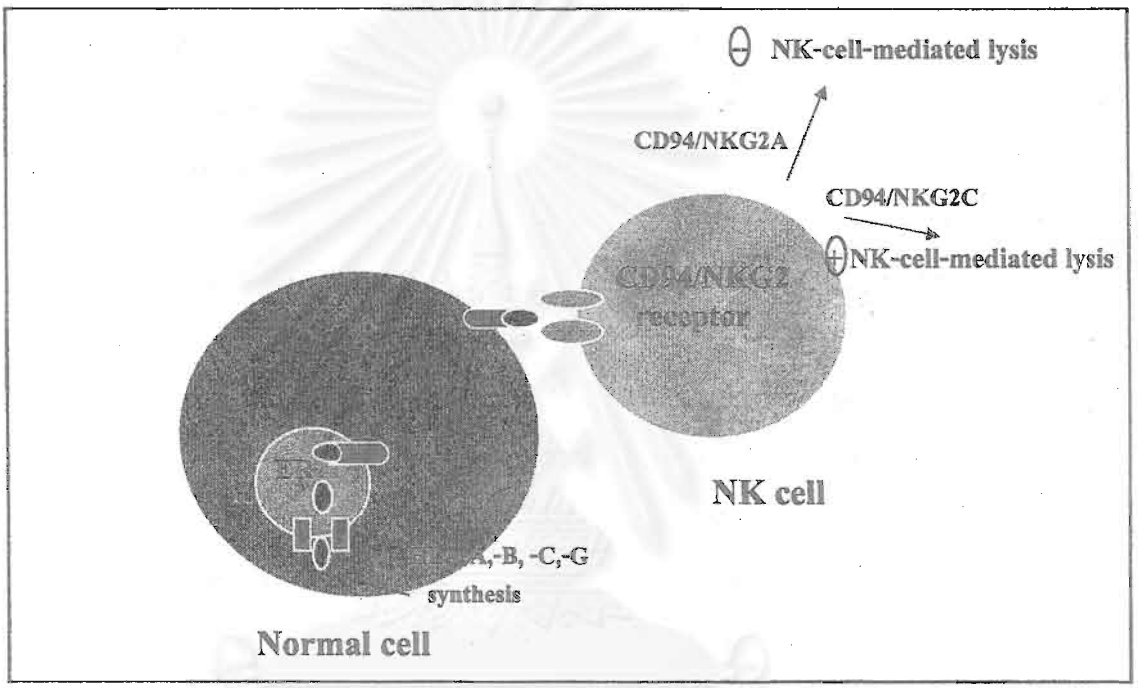


Figure 11 The proposed mechanism of HLA-E in NK cell function (O'Callaghan, 2000).

9. The role of HLA-E in cytotoxic T lymphocytes (CTLs)

HLA-E cell surface expression is dependent on acquisition of peptides derived from the leader sequences of other HLA class I molecules (Braud et al., 1997; Braud et al., 1998; Lee et al., 1998). Such HLA-E peptide complexes contribute the only known ligands for monomorphic CD94/NKG2 lectin-like heterodimers and affect only NK cell activity (Braud et al., 1998; Borrego et al., 1998; Maier et al., 2000). However, several recent observations raise the possibility that HLA-E might also plays an important role in the regulation of CTL function, as CD94/NKG2 receptors are expressed on a subset of CTL cells and their engagement may also critically influence CTL cell function (Mingari et al., 1998; Carena et al., 1997; Le Drean et al., 1998). Previous studies had noted that a small fraction of normal T cells bearing either TCRαβ or TCRγδ expresses KIRs or CD94 on the cell surface (Moretta et al., 1990; Aramburu et al., 1991). Additional analyses indicated that the majority of this T cell subset is $CD8^+$ and has a phenotype consistent with memory T cells (Mingari, 1996; Mingari, 1997). Furthermore, cross-linking KIRs or CD94/NKG2 strongly inhibits CTL-mediated cytotoxicity (Ferrini et al., 1994; Mingari et al., 1995; Phillips et al., 1995). These observations raise the possibility that inhibitory MHC-recognizing receptors could contribute to the down-regulation of a CTL response.

In addition, there is evidence that HLA-E can interact with T cell receptors (TCRs) directly. Qa-1b, a mouse homologue of HLA-E has been proposed to present H-2-unrelated antigenic peptides that may be specifically recognized by T lymphocytes (Bouwer et al., 1998; Jiang et al., 1998; Lowen et al., 1993; Lo et al., 2000; Jiang and Chess, 2000). In 1998, Ulbrecht et al. described that two peptide from influenza and Epstein-Barr virus proteins were shown to stabilize some cell-surface HLA-E molecules at 26 °c when they were added exogenously (Ulbrecht et al., 1998). Viral antigen might therefore be presented by HLA-E to T cell in a similar way to classical HLA class Ia molecules. A first indication that T cells may respond to HLA-E was obtained in a heterogous system based on the use of HLA-E transgenic mice (Pacasova et al., 1999). The results showed that the HLA-E/class I signal sequence derived peptides complex

was not only a ligand for NK cell inhibitory receptors, but can also interact with mouse TCRs (Martinozzi et al., 1999). Other have also reported that HLA-E can physically bind certain human TCR Vβ-derived peptides, and that HLA-E/ Vβ-peptide complexes can induce the differentiation of $CD8^+$ CTL, which preferentially lyse target in a TCR Vβ-specific manner (Li et al., 2001). A recently isolated T cell clone showed reactivity with self HLA-E. Staining of this clone with HLA-E tetramers could be inhibited by clonotypic anti-TCR antibodies. The data formally prove the existence of human T cells able to interact through their αβTCR with HLA-E molecules bound to class I-derived and foreign peptides (Garcia et al., 2002).

CHAPTER IV

MATERIALS AND METHODS

1. Subjects

In this study, the genomic DNA of NPC patients and control subjects extracted from peripheral blood leukocytes by standard phenol-chloroform extraction procedure as previously described (Maniatis, Fritsch and Sambrook, 1989) were obtained from previous work of Narisorn Kongrattanachoat (Narisorn Kongrattanachoat, 2001). The subjects include one hundred and seventy-four patients with NPC who attended at King Chulalonkorn Memorial Hospital from 1994 to 2001 and two hundred control samples from healthy blood donors of Thai Red Cross Society. This study has been approved by The Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The patients and controls were interviewed and then divided into three groups, Thai, Chinese, and Thai-Chinese, respectively, base on the ethnic origins of their grandparents. If their ancestors, including their great grandparents, originated from China, they were considered Chinese. On the other hand, if their ancestors originated from Thailand, they were defined as Thai. In addition, if their ancestors originated from Thailand and China, they were defined as Thai-Chinese.

The tumor of every patient was histologically ascertained as NPC type II or III, according to WHO classification. Ages of these patients ranged from 5 to 82 years (mean 46.8 years) at the time of diagnosis whereas the control subject ages ranged from 17-59 years (mean 35.5 years). Males outnumber females among the cases was 1.6: 1 and among the controls 1.7: 1, respectively. Number of DNA samples from the NPC patients and normal controls dividing by their ethnic were shown in Table 1.

Table 1 Number of DNA samples from the NPC patients and normal controls dividing by their ethnic.

Ethnic	NPC patients N=174 (%)	Normal controls N=200 (%)
Thai	104 (59.77)	100 (50)
Chinese	44 (25.29)	50 (25)
Thai-Chinese	26 (14.94)	50 (25)

2. PCR Amplification of the HLA-E Gene

The genomic DNA of one hundred and seventy-four NPC patients and two hundred healthy controls were amplified by HLA-E specific primers HLA-E.2F and HLA-E.2R described in Hodgkinson et al. (2000) which is specifically amplify exon 2 (Hodgkinson, Millward and Demaine, 2000). While primers HLA-E.3F and HLA-E.3R described in Gomez-Casado et al (1997) specifically amplify exon 3 (Gomez-Casado et al., 1997). The primer sequences used for amplification of the HLA-E gene were shown in Table 2.

Table 2 Primers used for amplification of the HLA-E gene and sizes of PCR products.

Primers	Sequence (5'→3')	size of PCR product (bp)
Exon 2		
HLA-E.2F	GAA ACG GCC TCT ACC GGG AGT AG	477
HLA-E.2R	GTT CCG CAG CCT TGG GGT GAA TC	
Exon3		
HLA-E.3F	CGG GAC TGA CTA AGG GGC	311
HLA-E.3R	AGC CCT GTG GAC CCT CTT	

The reaction volume for the amplification reaction was 50 μl, containing 50 ng/μl genomic DNA, 0.25 μl of 5.0 U Taq polymerase (Promega or Gibco), 5 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM deoxynucletide triphosphates and 2.5 μl (20 pmol) of each primer. Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The PCR protocol consisted of an initial denaturation at 94°c for 2 minutes, followed by 30 cycles of denaturation (94°c, 20 seconds), annealing (58°c, 50 seconds) and extension (72°c, 20 seconds) and final extension at 72°c for 7 minutes. The products were further analyzed by electrophoresis in 1.5 % Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 100 volts for 40 minutes and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD). The positive results of exon 2 or exon 3 of HLA-E gene showed a single band of 477 or 311 bp fragment compared with the 100-bp molecular size marker. Additionally, 10 selected PCR products were subsequently analyzed by DNA sequencing to confirm for HLA-E. To compare a nucleotide query sequence against a nucleotide sequence database, the sequences was analyzed by BLAST program (National Center for Biotechnology Information [NCBI], 1988).

3. SNPs finding

The positions of SNP on the HLA-E gene were searched from ASHI (American Society for Histocompatibility and Immunogenetics [ASHI], 2001). Seven positions of SNPs were found from ASHI. Four SNPs are on exon 2, codon2C/T, codon77C/T, codon82C/G and codon83G/C whereas three SNPs are on exon 3, codon107A/G, codon150C/T and codon157A/G. The positions of SNP on the HLA-E gene were summarized in Table 3. Besides searching database, the sequencing data from PCR products of HLA-E were also used to search for additional SNP in Thai population. However, out of 10 samples, no additional polymorphism at other positions was detected.

Table 3 The SNPs on exon 2 and exon 3 of HLA-E gene.

HLA-E alleles	Exon 2				Exon 3		
	Codon						
	2	77	82	83	107	150	157
HLA-E*0101	C	C	C	G	A	C	A
HLA-E*0102	C	C	G	C	A	C	A
HLA-E*01031	C	C	C	G	G	C	A
HLA-E*01032	C	T	C	G	G	C	A
HLA-E*01033	C	T	C	G	G	T	A
HLA-E*0104	T	C	C	G	G	C	G

The positions of SNP are on the HLA-E molecule, which are mainly lying on the peptide-binding groove. Four positions, codon2C/T, 77C/T, 82C/G and 150C/T are silence mutation. While three positions, codon83G/C, 107A/G and 157A/G are missense mutations, which change amino acids from glycine to arginine, arginine to glycine and arginine to glycine, respectively. The positions of SNP on HLA-E molecule were shown in Figure 12.

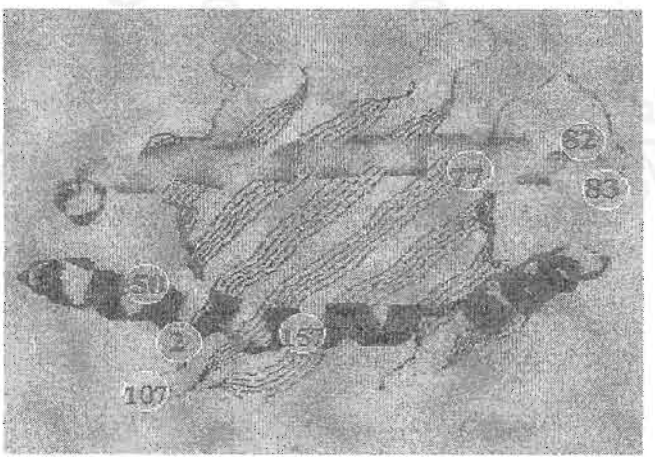


Figure 12 The positions of SNP on HLA-E molecule (Gomez-Casado et al., 1997).

4. SNPs detecting by dot blot hybridization

Twelve SSOPs (Sequence Specific Oligonucleotide probes) (Gamez-Casado et al., 1997) were used to detect SNPs of HLA-E gene as shown in Table 4. Two samples that were confirmed by sequencing were used as positive controls. PCR product from the amplification were subjected to SNP detection by dot blot hybridization as follow:

4.1 Dot blotting DNA

The nylon membrane (Hybond ^{TM}-N+; Amersham) was cut to a desired surface area and was prewetted with 10x SSC. The PCR products were denatured at 95° c for 2 minutes then chilled the samples on ice immediately. After that 20x SSC was added, mixed thoroughly and spinned briefly. The samples were applied onto the prewetted membrane, in approximately 2 µl aliquots, and allowed drying between each aliquot. The nylon membrane was wetted in denaturing solution for 5 minutes on a filter paper, then transferred the nylon membrane to a filter paper which was soaked with neutralizing solution for 1 minute. When the blot dry, DNA was fixed to the nylon membrane by baking in an oven at 80° c for 2 hours.

4.2 Preparation of SSOPs

Twelve SSOPs (Sequence Specific Oligonucleotide probes) were used to detect SNPs of HLA-E gene. Ten SSOPs previously described in Gamez-Casado et al (1997). Whereas two SSOPs of codon150C/T were designed in this study. The SSOPs were labeled with γ-^{32}P-ATP. The component of SSOPs labeling was shown in Table 5. Then γ-^{32}P-ATP labeled-SSOPs were incubated at 37° c for 1 hour.

Table 4 SSOPs used in this study.

	Probe	Codon	Sequence (5'→3')	HLA-E specificities
Exon 2	E2011	2	GGCTCCCACTCCTTG	0101, 0102, 01031, 01032, 01033
	E2012		GGCTCTCACTCCTTG	0104
	E2021	77	CGAGTGAACCTGCGG	0101, 0102, 01031, 0104
	E2022		CGAGTGAATCTGCGG	01032, 01033
	E2031	82, 83	CTGCGCGGCTACTAC	0101, 01031, 01032, 01033, 0104
	E2032		CTGCGGCGCTACTAC	0102
Exon 3	E3011	107	CCCGACAGGCGCTTC	0101, 0102
	E3012		CCCGACGGGCGCTTC	01031, 01032, 01033, 0104
	150C	150	GATGCCTCTGAGGCG	0101, 0102, 01031, 01032, 0104
	150T		GATGCTTCTGAGGCG	01033
	E3021	157	CACCAGAGAGCCTAC	0101, 0102, 01031, 01032, 01033
	E3022		CACCAGGGAGCCTAC	0104

Table 5 The component of SSOPs labeling.

Component	Volume/reaction (μl)	Concentration in mixture
10x kinase buffer	1	1x
T4 polynucleotide kinase (New English BioLab)	1	10U
γ-^{32}P-ATP (Amersham)	2	2mCi/ml
SSOPs (5 μM)	5	2.5 μM
Deionized H_2O	1	-
Total volume	10	

4.3 Setting up hybridization

The nylon membrane was placed in a hybridization tube. Prehybridization of the membrane was performed in a hybridization oven (Stuart Scientific) for 15 minutes at 42°c, in 10 ml of hybridization solution per 100 cm^2 of membrane (5x standard saline citrate [SSC], 1% blocking agent, 1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulfate [SDS]). Hybridization was carried out at 42°c for 1 hour with γ-^{32}P-ATP labeled-SSOPs. Posthybridization washing was done as follow: 5xSSC at 42°c for 10 minutes and twice for 10 minutes at 50°c in the same solution. The washing solution was drained off. The nylon membrane was removed and then was wrapped with Saran Wrap.

4.4 Detection of hybridization

The blots DNA side up were placed in a film cassette. A sheet of phosphor screen was placed on the top of the blots. The cassette was closed and exposed for 5-12 hours at room temperature. The sheet of phosphor screen was then removed and visualized on PhospholImager using ImageQuaNT Software (Molecular Dynamics).

5. DNA sequencing

As mention above, sequencing was used to confirm the specificity of PCR amplification as well as to screen for additional polymorphism at the other locations besides the one previously characterized. In addition, this method was used to confirm the ambiguous PCR-SSOPs results from heterozygous samples. For direct cycle sequencing, approximately 100 ng each of the PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN Inc.) to obtain clean double-standed DNA amplificates. Cycle sequencing was performed on an ABI Prism 310 Genetic Analyzer using a cycle sequencing chemistry with base-specific fluorescence – labeled dideoxynucleotide termination reagents. BigDye Terminator Ready Reaction Mix (Applied Biosystems) was used for sequencing. Thus, each sequencing reaction

mixture of 10 µl final volume contained 1 µl of 5 pmol primer, 3 µl of template and 3 µl of the BigDye Terminator Ready Reaction Mix. Each sample mixture was then subjected to a cycle sequencing reaction in a Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The condition of cycle sequencing reaction consisting of denaturation at 96° c for 30 seconds, annealing at 55° c for 10 seconds and extension at 60° c for 4 minutes were carried out. Then each sequencing reaction product was pooled into 2 µl of 3 M sodium acetate (NaOAc), pH4.6/50 µl of 95% ethanol (EtOH) mixture in 1.5 microcentrifuge tubes, incubated at room temperature for 15 minutes to precipitate the extension products and centrifuged at 13,000 rpm for 20 minutes. The products were washed with 70% ethanol (EtOH) and centrifuged for 5 minutes at 13,000 rpm. The DNA pellet was then dried by place the tubes with the lids open in a heat block or thermal cycler at 90° c for 1 minute. Finally, the samples were resuspended in 15 µl of TSR (template suppression reagent), heat the samples at 95° c for 2 minutes and then chill on ice. The samples were loaded into an ABI Prism 310 Genetic Analyzer. Data collection was performed using the software package provided with the ABI 310 a sequencing system.

6. Statistical analysis

Allele frequencies in the population samples were determined by direct gene counting. The association between certain alleles of HLA-E and NPC development was estimated by the statcalc from Epi info version 6 to calculate the odds ratio (OR) and 95% confidence interval (CI), Mantel-Haenszel chi squares and associated p values. A level of $P<0.5$ was accepted as statistically significant. Mantel-Haenszel weighted OR, summary chi square and p values were adjusted for the confounding effect of ethnic by combination stratified 2X2 tables (Centers for Disease Control and Prevention [CDC], 1994). The effect exerted by genotype was estimated as if autosomal inheritance according to actual number of alleles contributed to a significant OR.

Haplotype frequencies were estimated by the Estimating Haplotype-frequencies (EH) software program and Pascal compiler (Zhao, Curtis and Sham, 2000; Laplace, 2000). The number of cases with haplotype CA/TG or CG/TA were estimated according to the probability of each combination. The calculation was performed according to these formulas CA/TG = N x [EH(CA)xEH(TG)]/{[EH(CA)xEH(TG)]+[EH(CG)xEH(TA)]} and CG/TA = N x [EH(CG)xEH(TA)]/{[EH(CG)xEH(TA)]+[EH(CA)xEH(TG)]}. N is number of cases with compound herterozygote. EH (haplotype) is haplotype frequency from EH calculation of each indicated haplotype. Haplotype alleles from HLA-E77C/T and 107A/G composed of four forms, CA, CG, TA and TG. Moreover, the association between each haplotype and NPC development was calculated based on the OR uses Epi info version 6 program (Centers for Disease Control and Prevention [CDC], 1994). The haplotype differences between groups were estimated by T (χ^2/2) = ln (L, group1) + ln (L, group2) - ln (L, group1+ group2) as previously described (Zhao et al., 2000).

CHAPTER V

RESULTS

1. Specific amplification of the HLA-E gene

Specific amplification of the HLA-E gene was successfully achieved by PCR with specific primers for exon 2 and exon 3. All samples gave positive PCR results, which subsequently were used for SNPs detection by dot blot hybridization. The representative results of PCR-amplified products of the HLA-E gene were shown in Figure 13.

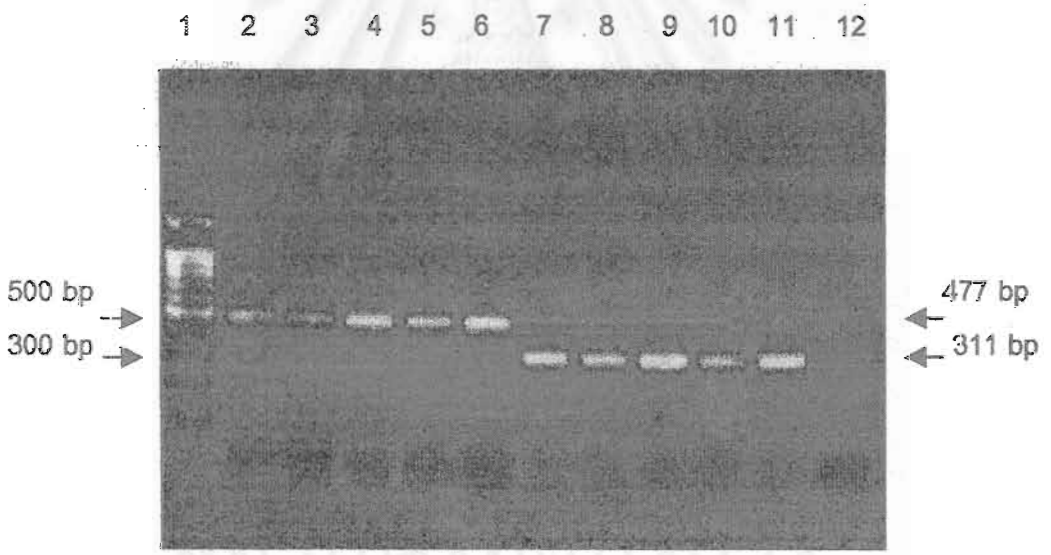


Figure 13 The representative of PCR-amplified products of exon 2 and exon 3 of the HLA-E gene.

Lane 1 : 100 bp molecular marker

Lane 2-6 : PCR-amplified products of exon 2 ; size of DNA fragment approximately 477 bp

Lane 7-11 : PCR-amplified products of exon 3 ; size of DNA fragment approximately 311 bp

Lane 12 : Negative control

2. Confirmation of the PCR-positive results of the HLA-E gene

Ten PCR products of exon 2 and exon 3 of the HLA-E gene were analyzed by DNA sequencing. Then the BLAST program was used to compare a nucleotide sequence against the sequence database. The PCR products were confirmed to be derived from HLA-E as shown in Figure 14.

In addition, DNA sequencing was used to screen for additional polymorphism at the other locations besides the one previously characterized. In this study, sequence results did not reveal any new polymorphism at the other locations.

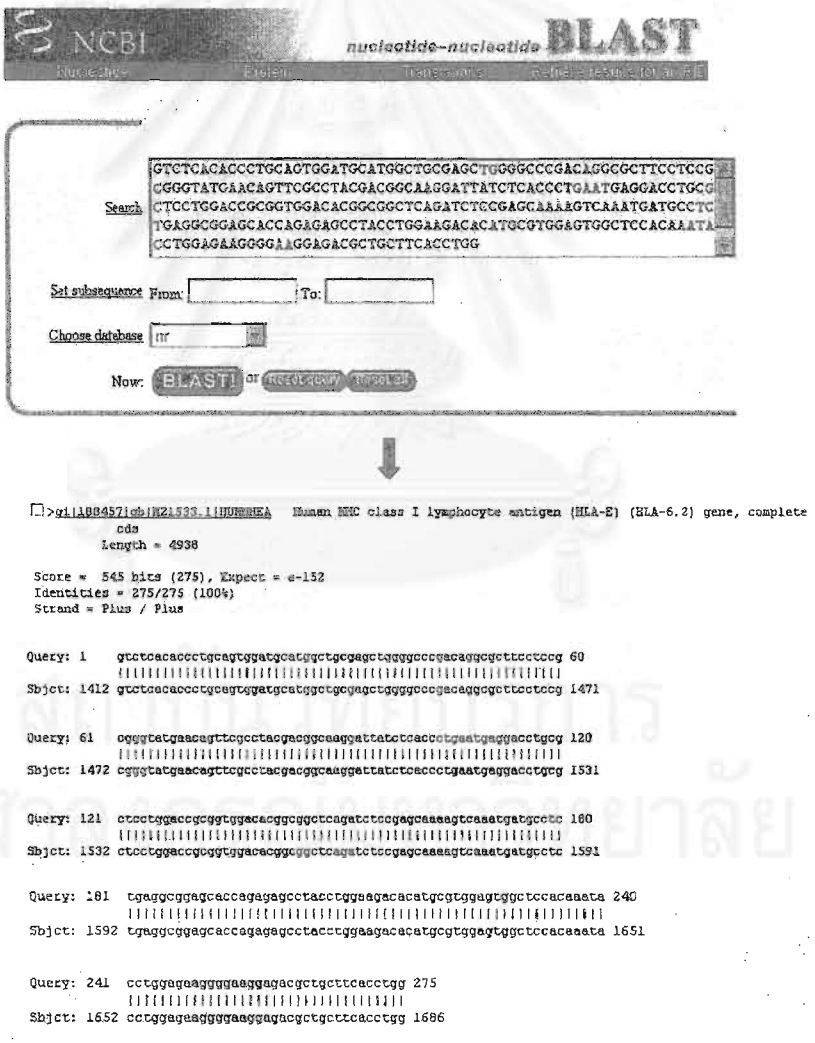
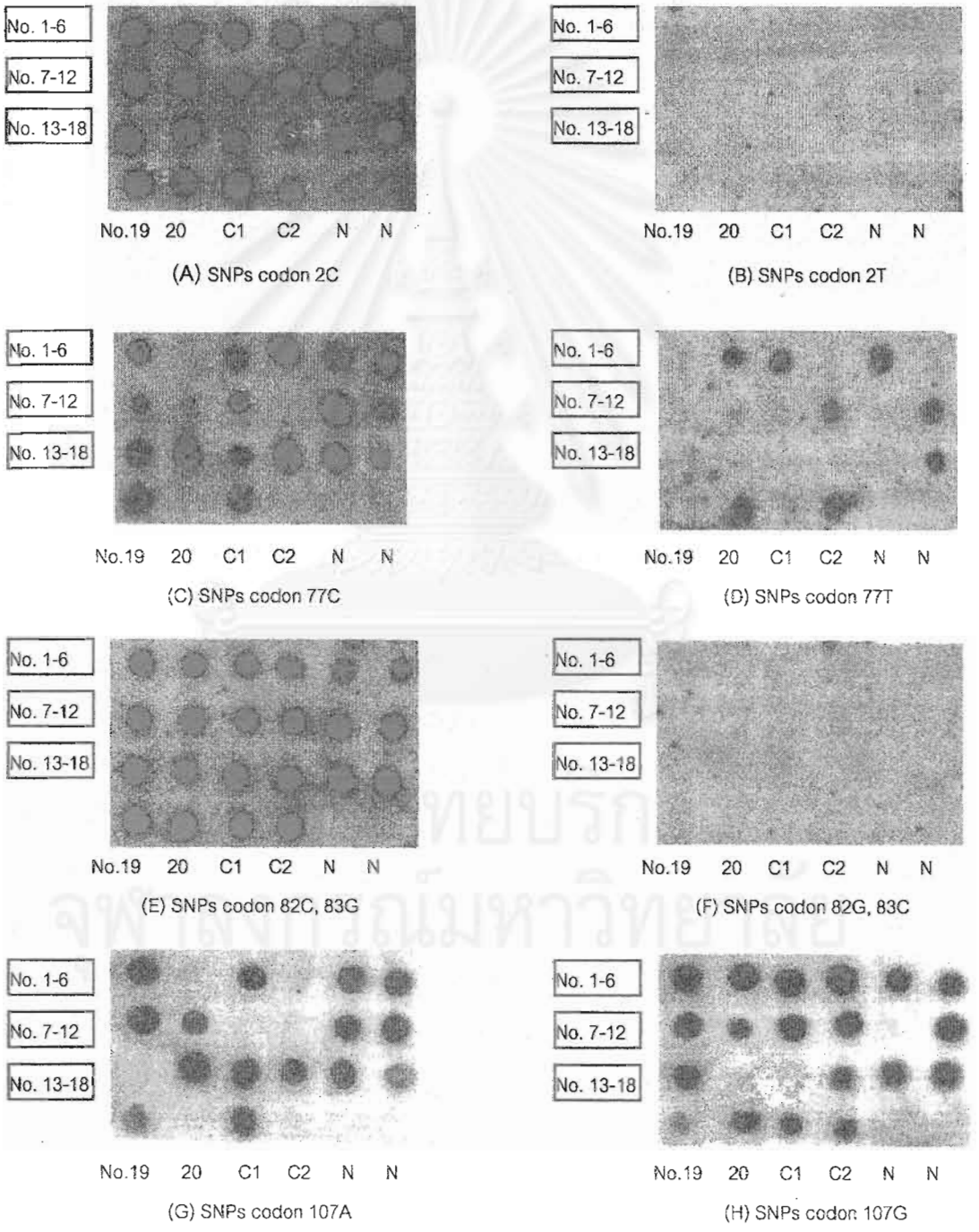


Figure 14 Confirmation of the direct sequencing of the PCR fragment derived from the HLA-E gene by BLAST program.

3. SNPs analysis by dot blot hybridization

PCR-amplified products of all samples were typed by dot blot hybridization. Twelve SSOPs were used to detect 7 SNPs; codon2C/T, 77C/T, 82C/G, 83G/C, 107A/G, 150C/T and 157A/G. The representatives of SNPs analysis of HLA-E are demonstrated in Figure 15.



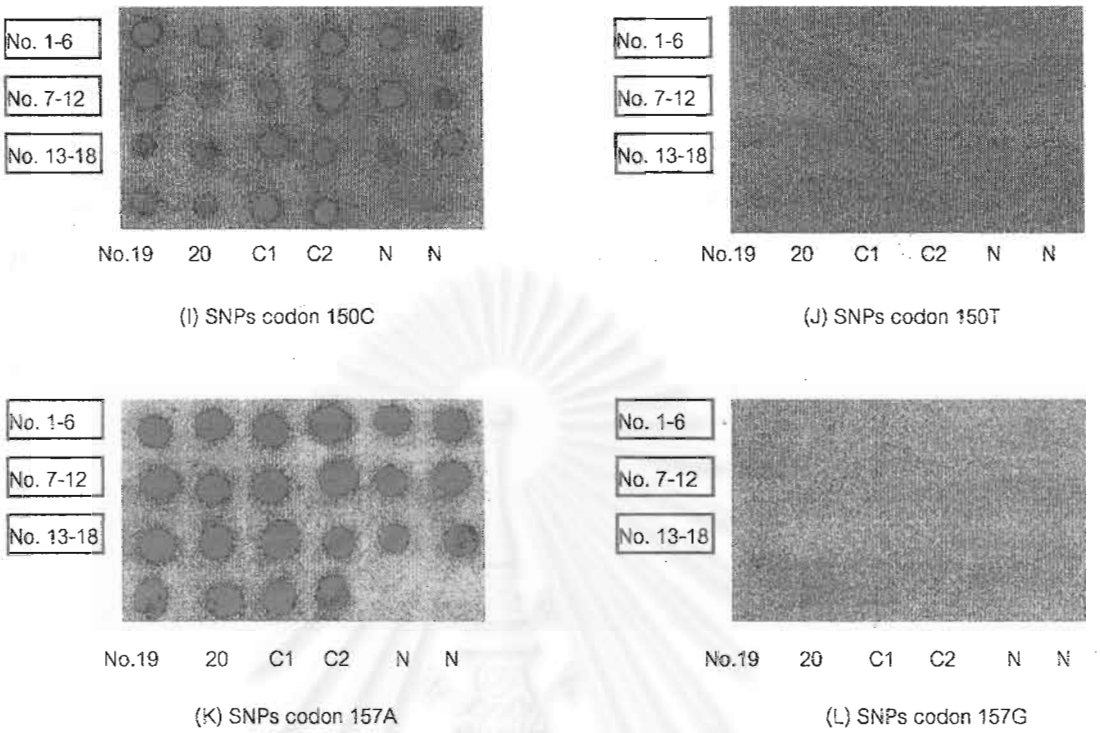
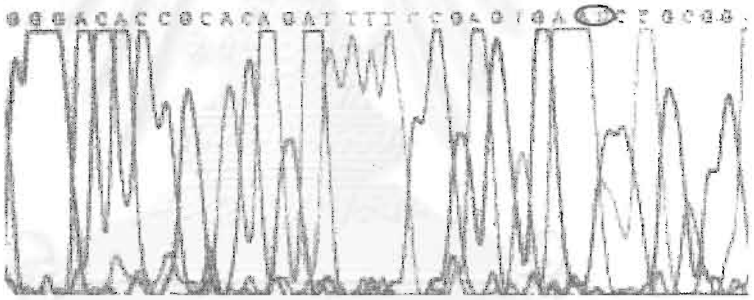


Figure 15 The representatives of SNPs analysis of the HLA-E gene by dot blot hybridization. (A,B,E,F,I,J,K and L) N represented negative control, C1 and C2 were the positive controls for HLA-E2C, 82C, 83G, 150C and 157A. All samples were homozygous 2C/C, 82C/C, 83G/G, 150C/C and 157A/A. (C,D) For HLA-E77C/T, C1 was the positive control for homozygous C/C, C2 was the positive control for homozygous T/T. Samples 1,4,6,7,9,11,13-17 and 19 were homozygous C/C, samples 2,10 and 20 were homozygous T/T and samples 3,5,8,12 and 18 were heterozygous C/T. (G,H) For HLA-E107A/G, C1 was the positive control for heterozygous A/G, C2 was the positive control for homozygous G/G. Sample 1,3,5-8,12,16-18 and 19 were heterozygous A/G, samples 11,14 and 15 were homozygous A/A and samples 2,4,9,10,13 and 20 were homozygous G/G. Some samples (no.8 (HLA-E77) and 19 (HLA-E107)) from dot blot hybridization showed the ambiguous results, which were investigated repeatedly by DNA sequencing.

4. DNA sequencing results used for confirming the heterozygous results

DNA sequencing was also used to confirm the heterozygous results from dot blot hybridization method. Exon 2 and exon 3 which HLA-E codon 77C/T and 107A/G located were amplified from genomic DNA samples of 19 NPC patients and 10 controls. This amplification using two pairs of primers as previously described (Hodgkinson et al., 2000; Gomez-Casado et al., 1997). PCR were expected to yield amplicons of 477 bp and 311 bp. The nucleotide sequences were determined by automate sequencing method as previously described. The representatives of DNA sequencing results were illustrated in Figure 16.

(A)



(B)

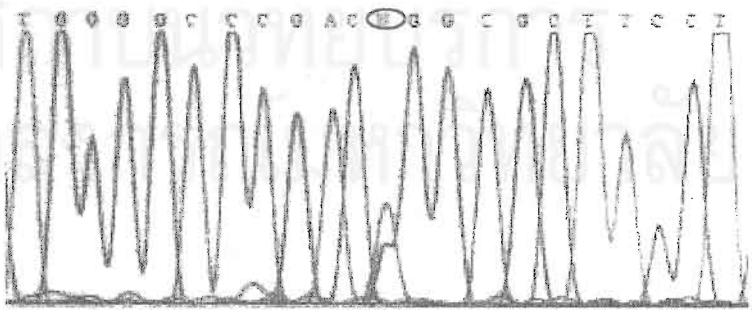


Figure 16 The representatives of chromatogram were obtained from automate sequencing showed heterozygous C/T from sample 8 at position HLA-E77C/T (see figure 15 C,D) (A), heterozygous A/G from sample 19 at position HLA-E107A/G (see figure 15 G,H) (B).

5. The results of SNPs analysis

5.1 Genotype frequencies of the HLA-E polymorphism

SNPs of the HLA-E gene were genotyped by dot blot hybridization. Polymorphism could be found only at codon 77C/T and 107A/G in this study whereas polymorphism at codons 2C/T, 82C/G, 83G/C, 150C/T, 157A/G were not found in this study. The genotype frequencies of the HLA-E polymorphism in NPC patients and controls at the SNP codon 77 and 107, divided into three groups; Thai, Chinese and Thai-Chinese were reported in Table 6. The homozygous CC at codon 77C/T was the most common genotype in Thai patients (48.08%) and controls (58%) followed by CT and TT genotypes. The CT was the most common genotype in Chinese patients (61.36%) and controls (50%), followed by CC and TT genotypes. The HLA-E genotype patterns of Thai-Chinese patients are similar to the patterns of Thai origin, occurring with a frequency of 58% for CC, 26.92% for CT and 23.08% for TT, respectively. However, Thai-Chinese controls differed from patients, the CC and CT were the predominant genotypes, followed with the TT genotype that found in only 8%.

At the codon 107A/G, the GG genotype was the most common in NPC patients of all ethnic groups (Thai, Chinese and Thai-Chinese), occurring with a frequency of 50.96%, 45.45% and 50%, respectively. The AG was the second most common genotype in Thai (39.42%), Chinese (40.91%) and Thai-Chinese patients (38.46%), whereas the genotype frequencies of AA comprised 9.62% for Thai, 13.64% for Chinese and 11.54% for Thai-Chinese. In contrast to NPC patients, the AG was the predominant genotype in Thai (49%) and Thai-Chinese controls (46%), followed by GG and AA. Among Thai controls, the genotype frequencies are 33% for GG and 18% for AA, whereas Thai-Chinese controls showed the genotype frequencies of 40% for GG and 14% for AA. Among Chinese, the GG was the most common genotype with a frequency of 46%, followed by AG (44%) and AA (10%).

Table 6 Genotype frequencies of HLA-E polymorphism in NPC patients and controls at the SNP codon 77 and 107 dividing by their ethnic.

SNPs n (%)	ETHNIC	THAI n (%)	CHINESE n (%)	THAI-CHINESE n (%)	TOTAL
77	NPC patients				
	CC	50 (48.08)	14 (31.82)	13 (50)	77 (44.25)
	CT	41 (39.42)	27 (61.36)	7 (26.92)	75 (43.1)
	TT	13 (12.5)	3 (6.82)	6 (23.08)	22 (12.64)
	Controls				
	CC	58 (58)	23 (46)	23 (46)	104 (52)
	CT	35 (35)	25 (50)	23 (46)	83 (41.5)
	TT	7 (7)	2 (4)	4 (8)	13 (6.5)
107	NPC patients				
	AA	10 (9.62)	6 (13.64)	3 (11.54)	19 (10.92)
	AG	41 (39.42)	18 (40.91)	10 (38.46)	69 (39.66)
	GG	53 (50.96)	20 (45.45)	13 (50)	86 (49.43)
	Controls				
	AA	18 (18)	5 (10)	7 (14)	30 (15)
	AG	49 (49)	22 (44)	23 (46)	94 (47)
	GG	33 (33)	23 (46)	20 (40)	76 (38)

5.2 Association between HLA-E SNPs at codon 77 and NPC

The allele frequencies of HLA-E SNP patterns between NPC patients and controls were compared among the identical ethnic origin, Thai, Chinese and Thai-Chinese. HLA-E77T allele demonstrated no considerable correlation with NPC when analyzing in each ethnic group. But when evaluating all three groups together, a weak associated OR of 1.39 (95% CI = 1.00-1.92, p = 0.04) was found. The significant ethnic groups adjusted OR was 1.42 (95%CI = 1.02-1.97, p = 0.036) (Table 7).

The effect of HLA-E77T was similar to autosomal recessive mode of inheritance. The presence of two T alleles (TT) conferred the significant OR of 2.08 (95% CI = 0.96-4.54, p = 0.042) and the race adjusted OR was 2.20 (95% CI = 1.02-4.95, p = 0.046) (Table 8).

5.3 Association between HLA-E SNPs at codon 107 and NPC

Analysis of SNP patterns of codon 107 showed a significant association in Thai origin between NPC and HLA-E107G allele with OR of 1.78 (95% CI = 1.16-2.74, p=0.006). However, no significant association of this allele was found in other populations (Table 9). When we analyzed all three ethnic groups, the crude OR of HLA-E107G was 1.41 (95% CI = 1.03-1.94, p=0.026), whereas the ethnic group adjusted OR was 1.42 (95% CI = 1.04-1.96, p=0.029) (Table 9).

The NPC susceptibility among Thai origin conferred by HLA-E107G is in consistent with autosomal recessive mode of inheritance in that two alleles are required to increase the likelihood of NPC development, with OR of 2.11 (95%CI = 1.15-3.88, p = 0.009) (Table 10). When including three ethnic groups together, the slightly significant OR was 1.59 (95% CI = 1.03-2.46, p = 0.026) and the significant race adjusted OR was 1.61 (95% CI = 1.04-2.51, p = 0.031) (Table 10).

Table 7 Allele frequencies of HLA-E polymorphism at codon 77 between NPC patients and controls according to their grandparents' ethnic origin.

	THAI		CHINESE		THAI-CHINESE		TOTAL	
	case	control	case	control	case	control	case	control
Number of alleles	208	200	88	100	52	100	348	400
HLA-E77C/T								
C	141	151	55	71	33	69	229	291
T	67	49	33	29	19	31	119	109
T allele frequencies	0.32	0.25	0.38	0.29	0.37	0.31	0.34	0.27
Crude OR(95%CI)	1.46(0.93-2.31)		1.44(0.75-2.78)		1.28(0.60-2.75)		1.39(1.00-1.92)[a]	
Ethnic group adjusted OR(95%CI)							1.42(1.02-1.97)[b]	

C and T are nucleotide at codon 77.

OR (95% CI) = odd ratio and 95% confidence interval of HLA-ET allele when compared with the HLA-EC allele.

[a] $p = 0.04$, [b] $p = 0.036$.

Table 8 Risk of nasopharyngeal carcinoma associated with HLA-E77C/T genotype according to different models of inheritance.

	THAI OR(95%CI)	Chinese OR(95%CI)	THAI-CHINESE OR(95%CI)	TOTAL OR(95%CI)	ETHNIC GROUP ADJUSTED OR(95%CI)
T codominance, C wild type					
CC	1.00	1.00	1.00	1.00	1.00
CT	1.36(0.72-2.56)	1.77(0.69-4.59)	0.54(0.16-1.80)	1.22(0.78-1.92)	1.24(0.78-1.98)
TT	2.15(0.73-6.54)	2.46(0.28-24.72)	2.65(0.52-14.15)	2.29(1.02-5.15)[a]	2.32(1.03-5.30)[b]
T dominance, C wide type					
CC	1.00	1.00	1.00	1.00	1.00
TT or CT	1.49(0.83-2.69)	1.83(0.72-4.64)	0.85(0.30-2.44)	1.36(0.89-2.10)	1.41(0.91-2.18)
T recessive, C wide type					
CT or CC	1.00	1.00	1.00	1.00	1.00
TT	1.90(0.67-5.54)	1.76(0.22-15.94)	3.45(0.75-16.72)	2.08(0.96-4.54)[c]	2.20(1.02-4.95)[d]

CC and TT are the homozygous C and T at codon 77, respectively, CT is the heterozygous at codon 77.

OR(95%CI) is odd ratio and 95% confidence interval between allele and compared allele, [a]p=0.027, [b]p=0.041, [c]p=,0.042, [d]p=0.046.

Table 9 Allele frequencies of HLA-E polymorphism at codon 107 between NPC patients and controls according to their grandparents' ethnic origin.

	THAI		CHINESE		THAI-CHINESE		TOTAL	
	case	control	case	control	case	control	case	control
HLA-E107A/G								
A	61	85	30	32	16	37	107	154
G	147	115	58	68	36	63	241	246
G allele frequencies	0.71	0.58	0.66	0.68	0.69	0.63	0.69	0.62
Crude OR(95%CI)	1.78(1.16-2.74)[a]		0.91(0.47-1.75)		1.32(0.61-2.88)		1.41(1.03-1.94)[b]	
Ethnic group adjusted OR(95%CI)							1.42(1.04-1.96)[c]	

A and G are nucleotide at codon 107.

OR (95% CI) = odd ratio and 95% confidence interval of HLA-EG allele when compared with the HLA-EA allele.

[a] p = 0.006, [b] p = 0.026, [c] p = 0.029.

Table 10 Risk of nasopharyngeal carcinoma associated with HLA-E107A/G genotype according to different models of inheritance.

	THAI OR(95%CI)	CHINESE OR(95%CI)	THAI-CHINESE OR(95%CI)	TOTAL OR(95%CI)	ETHNIC GROUP ADJUSTED OR(95%CI)
G codominance, A wild type					
AA	1.00	1.00	1.00	1.00	1.00
GG	2.89(1.10-7.73)[a]	0.72(0.16-3.26)	1.52(0.27-9.15)	1.79(0.89-3.61)	1.80(0.90-3.74)
GA	1.51(0.58-3.97)	0.68(0.15-3.12)	1.01(0.18-6.26)	1.16(0.57-2.35)	1.16(0.57-2.35)
G dominance, A wide type					
AA	1.00	1.00	1.00	1.00	1.00
GG or GA	2.06(0.84-5.12)	0.70(1.17-2.88)	1.25(0.25-6.81)	1.44(0.75-2.78)	1.45(0.75-2.83)
G recessive, A wide type					
GA or AA	1.00	1.00	1.00	1.00	1.00
GG	2.11(1.15-3.88)[b]	0.98(0.4-2.93)	1.50(0.52-4.35)	1.59(1.03-2.46)[c]	1.61(1.04-2.51)[d]

GG and AA are the homozygous G and A at codon 107, respectively, GA is the heterozygous at codon 107.

OR (95%CI) is odd ratio and 95% confidence interval between allele and compared allele, [a] p=0.017, [b] p=0.009, [c] p=0.026, [d] p=0.031.

6. The results of haplotype analysis

In addition to SNP marker data, haplotypes of markers were used for association analysis of candidate genes. Haplotypes are the particular combination of alleles at different sites on a single chromosome. Genotype data of 77C/T and 107A/G were tabulated into four haplotypes, 77C-107A(CA), 77C-107G(CG), 77T-107A(TA) and 77T-107G(TG) according to all possible combination of the two SNPs. Haplotype analysis was divided into two parts. First, the estimation of the frequency of each haplotype by EH program and comparison of the distribution of haplotype frequencies between groups by the formula as previous described (Zhao et al., 2000). Second, the analysis of the association between HLA-E haplotype and NPC development.

6.1 Estimation and distribution of the haplotype frequencies

The EH program was applied for the haplotype analysis in the present study and succeeded in estimating an approximate value of haplotype frequency using the entire data of genotypes. The overall distribution was shown in Table 11. The CA, CG and TG were the most frequent haplotype whereas TA was the least frequent haplotype in all groups. In addition, the distribution of the haplotype frequencies was compared between groups. There were relevant differences between Thai and Chinese NPC patients (χ^2 = 44.56, df = 3 and $p < 0.005$). Among the control, the distribution of haplotype frequencies differ significantly between Thai and Thai-Chinese (χ^2 = 155.24, df = 3 and $p < 0.005$). Furthermore, the distribution of the haplotype frequencies was compared between case and control of each ethnic group. There were significant difference in Thai and total between case and control with χ^2 = 9.58, df = 3 and $p < 0.025$ and χ^2 = 9.22, df = 3 and $p < 0.05$, respectively, but no significant difference in other populations.

Table 11 Haplotype frequencies of HLA-E77-107 from EH calculation.

	THAI		CHINESE		THAI-CHINESE		TOTAL	
Haplotype	case	control	case	control	case	control	case	control
CA	0.286147	0.424993	0.325721	0.319990	0.307688	0.369993	0.299476	0.384995
CG	0.391737	0.330007	0.299279	0.390010	0.326928	0.320007	0.358570	0.342505
TA	0.007122	0.000007	0.015188	0.000010	0.000005	0.000007	0.007996	0.000005
TG	0.314993	0.244993	0.359812	0.289990	0.365380	0.309993	0.333958	0.272495

Haplotypes were shown as combined of 77C/T and 107A/G.

Thai patients compared with Chinese patients $\chi^2 = 44.56$, $p < 0.005$.

Thai controls compared with Thai-Chinese controls $\chi^2 = 155.24$, $p < 0.005$.

Thai patients compared with Thai controls $\chi^2 = 9.58$, $p < 0.025$.

Total patients compared with total control $\chi^2 = 9.22$, $p < 0.05$.

6.2 Association between HLA-E haplotype and NPC development

To study the significance of each haplotype, the actual allele frequencies will be used to compare. In cases that the genotypes were homozygous at least for one marker, their exact genotypes could be directly tabulated. However, haplotype of people with compound heterozygous could not be determined because they would have two possible haplotypes from CT and AG genotypes, CA/TG and CG/TA. The estimate number was calculated from probability of haplotype frequency from EH calculation using the following formulas N x [EH(CA)xEH(TG)]/{[EH(CA)xEH(TG)]+[EH(CG)xEH(TA)]} and N x [EH(CG)xEH(TA)]/{[EH(CG)xEH(TA)]+[EH(CA)xEH(TG)]} for CA/TG and CG/TA, respectively. The haplotype frequencies of each ethnic group were shown in Table 12. As can be seen, the CA/TG haplotype combination was the most common whereas CG/TA was very rare haplotype combination. The number of each haplotype from each ethnic group was counted based on the allele frequencies multiplied by the number of case as listed in Table 13. To test hypothesis that HLA-E SNPs or another linked genetic mutation was responsible for NPC development. The number of tested haplotype and

the other three alleles between NPC patients and controls were compared among the identical ethnic origin. There were no haplotype revealed any considerable risk from all groups, but CA haplotype was displayed as protective in the Thai and the total population with OR of 0.55 (95% CI = 0.36-0.84, p=0.004) and OR of 0.69 (95% CI = 0.50-0.95, p= 0.017), respectively (Table 13). The variation of the significant relative risk of each haplotype might be due to the present of the same SNPs from another haplotype. Thus, the relative risk of each haplotype was reevaluated by excluding the other haplotype with the same 77 or 107 SNPs. For example, when comparing CG haplotype and the other haplotypes, the CA haplotype with the same 77C SNP was excluded. Both CG and TG were revealed as susceptible alleles. The significant OR of CG haplotype could be determined from Thai (OR = 1.71, 95% CI = 1.05-2.79, p= 0.023), whereas TG haplotype showed significant OR in Thai and total population with OR of 1.88 (95% CI = 1.11-3.18, p=0.012 and OR of 1.54 (95% CI = 1.06-2.25, p=0.02), respectively. The CA haplotype still showed as protective allele in the Thai and the total (Table 14). In addition, the relationship between each haplotype was measured and the data obtained supported the importance of the HLA-E107G allele. The OR between CG or TG and CA were statistically significant, but no significant OR was demonstrated between CG and TG (Table 14).

Table 12 Haplotype frequencies at codon 77C/T and 107A/G of the HLA-E gene.

	THAI		CHINESE		THAI-CHINESE		TOTAL	
HLA-E77-107	case	control	case	control	case	control	case	control
CC-AA C C | | A A	9	18	5	5	3	7	17	30
CC-AG C C | | A G	25	27	9	13	7	12	41	52
CC-GG C C | | G G	16	13	0	5	3	4	19	22
CT-AA C T | | A A	1	0	1	0	0	0	2	0
CT-GG C T | | G G	24	13	17	16	4	12	45	41
TT-GG T T | | G G	13	17	3	2	6	4	22	13
* CT-AG	16	22	9	9	3	11	28	42
(1) C T | | A G	16	22	9	9	3	11	27	42
(2) C T | | G A	0	0	0	0	0	0	1	0

* There are two possible combinations of haplotypes. The estimate number was calculated from probability of haplotype frequency from EH calculation using the following formulas N x [EH(CA)XEH(TG)]/{[EH(CA)XEH(TG)]+[EH(CG)xEH(TA)]} and N x [EH(CG)XEH(TA)]/{[EH(CG)XEH(TA)] + [EH(CA)xEH(TG)]} for CA/TG(1) and CG/TA(2), respectively.

Table 13 Haplotype number of HLA-E77-107 based on allele frequencies, crude OR and ethnic group adjusted OR.

	THAI		CHINESE		THAI-CHINESE		TOTAL		ETHINIC GROUP ADJUSTED
Haplotype	case	control	case	control	case	control	case	control	
CA	60	85	29	32	16	37	105	154	
OR (95%CI)	0.55(0.36-0.84)[a]		1.04(0.54-2.01)		0.76(0.35-1.64)		0.69(0.50-0.95)[b]		0.69(0.50-0.94)[c]
CG	81	66	26	39	17	32	124	137	
OR (95%CI)	1.29(0.85-1.98)		0.66(0.34-1.26)		1.03(0.47-2.24)		1.06(0.78-1.45)		1.05(0.76-1.43)
TA	1	0	1	0	0	0	2	0	
OR (95%CI)	undefined		undefined		undefined		undefined		undefined
TG	66	49	32	29	19	31	117	109	
OR (95%CI)	1.43(0.91-2.27)		1.40(0.72-2.70)		1.28(0.60-2.75)		1.35(0.98-1.87)		1.39(1.00-1.93)

OR (95%CI) = odd ratio and 95% confidence interval between the tested haplotype and the other three haplotypes. [a] p=0.004, [b] p=0.017 and [c] p=0.019.

Table 14 Odd ratio and 95% confidence interval of HLA-E77-107 haplotype when excluded interference haplotype and comparing between each haplotype.

HAPLOTYPE VS HAPLOTYPE	THAI OR(95%CI)	CHINESE OR(95%CI)	THAI-CHINESE OR(95%CI)	TOTAL OR(95%CI)	ETHINIC GROUP ADJUSTED OR(95%CI)
HLA-E77C					
CG/TG+TA	0.90(0.53-1.31)	0.59(0.27-1.26)	0.87(0.35-2.13)	0.83(0.57-1.20)	0.80(0.55-1.16)
CA/TG+TA	0.52(0.31-0.87)[a]	0.80(0.37-1.72)	0.71(0.29-1.73)	0.62(0.43-0.91)[b]	0.62(0.42-0.90)[c]
HLA-E77T					
TG/CG+CA	1.44(0.91-2.28)	1.42(0.74-2.76)	1.28(0.60-2.75)	1.36(0.99-1.89)	1.40(1.01-1.94)[d]
TA/CG+CA	undefined	undefined	undefined	undefined	undefined
HLA-E107A					
CA/CG+TG	0.55(0.36-0.85)[e]	1.06(0.55-2.05)	0.76(0.35-1.64)	0.70(0.51-0.96)[f]	0.69(0.50-0.95)[g]
TA/CG+TG	undefined	undefined	undefined	undefined	undefined
HLA-E107G					
CG/CA+TA	1.71(1.05-2.79)[h]	0.71(0.33-1.52)	1.23(0.49-3.06)	1.30(0.91-1.87)	1.29(0.90-1.87)
TG/CA+TA	1.88(1.11-3.18)[i]	1.18(0.55-2.54)	1.42(0.58-3.49)	1.54(1.06-2.25)[j]	1.57(1.08-2.30)[k]
CG/TG	0.91(0.54-1.54)	0.60(0.28-1.30)	0.87(0.35-2.13)	0.84(0.58-1.22)	0.81(0.55-1.18)
CG/CA	1.74(1.07-2.84)[l]	0.74(0.34-1.58)	1.23(0.49-3.06)	1.33(0.92-1.91)	1.32(0.92-1.91)
CG/TA	undefined	undefined	undefined	undefined	undefined
TG/CA	1.91(1.13-3.23)[m]	1.22(0.56-2.64)	1.42(0.58-3.49)	1.57(1.08-2.29)[n]	1.60(1.09-2.34)[o]
TG/TA	undefined	undefined	undefined	undefined	undefined
CA/TA	undefined	undefined	undefined	undefined	undefined

OR (95%CI) = odd ratio and 95% confidence interval between the tested haplotype and the other two haplotypes without the same SNPs and OR between each haplotype. [a] p=0.009, [b] p=0.01, [c] p=0.011, [d] p=0.044, [e] p=0.004, [f] p=0.02, [g] p=0.022, [h] p=0.023, [i] p=0.012, [j] p=0.017, [k] p=0.012, [l] p=0.019, [m] p= 0.01, [n] p= 0.013 and [o] p=0.015.

7. Diversity of HLA-E in Thailand

Besides SNP analysis, this study also provides the basic knowledge of the frequency of HLA-E alleles in population of Thailand. The determination of each allele depends on the presence of specific nucleotide as shown in Table 3. For example, the HLA-E*0101 allele presents with polymorphism at the codon 83 (G/C) and the codon 107(A/G) both consisting in missence substitutions. A nonsynonymous conservative change from glycine to arginine at position 83(G/C) and a synonymous change of the codon 82(C/G) define the HLA-E*0102. The two alleles from the HLA-E*0103 lineage correspond to HLA-E107G. A silent substitution (C/T) at codon 77 in exon 2 distinguished E*01032 from E*01031. The HLA-E*01033 allele corresponds to HLA-E150T. The HLA-E*0104 allele is defined by a silent substitution at codon 2 (C/T) or a nonsynonymous change from arginine to glycine at amino acid position 157 (A/G). In this study, three alleles of HLA-E could be detected on the basis of these polymorphisms. The HLA-E allelic frequencies (%) in three populations described in this study are shown in Table 15. The E*0101 is the most common allele in Thai and Thai-Chinese with allelic frequencies of 42.5% and 38%, respectively. The other HLA-E allele frequencies of Thai origin are 33% and 24.5% for E*01031 and E*01032, respectively. Among Thai-Chinese, the allele frequencies of HLA-E are 31% for both E*01031 and E*01032. Whereas the E*01031 was the predominant allele in Chinese origin with a frequency of 39%, followed by E*0101 and E*01032 with 32% and 29%, respectively. No E*01033, E*0102 and E*0104 could be detected in all individuals. When comparing the distribution of HLA-E alleles between each population (Thai vs Chinese, Thai vs Thai-Chinese and Chinese vs Thai-Chinese), no significant difference could be found among these populations.

In addition, the comparisons of the HLA-E alleles distribution between our populations and other populations previously reported were analyzed (Table 15). The analysis showed significant difference between each of our populations (Thai, Chinese and Thai-Chinese) and Danish population (χ^2=15.64, p=0.0004; χ^2=24.58, p=0.0000046; χ^2=14.69, p=0.00065), respectively. In addition, when compared to

African population, only Chinese origin showed a significant difference with χ^2 =7.61 and p=0.022. No significant difference of the distribution of HLA-E alleles between our populations and Shanghai Chinese or Japanese population could be detected.

Table 15 HLA-E allelic frequencies (%) in different populations

HLA-E allele	Thai[a] (n=200)	Chinese[b] (n=100)	Thai-Chinese[c] (n=100)	Danish (n=300)	Shanghai-Chinese (n=402)	Japanese (n=100)	Indigenous African (n=216)
0101	42.5	32	38	56.67	42.29	32	50.93
0102	0	0	0	0	0	0	0
01031	33	39	31	9.67	24.88	39	29.63
01032	24.5	29	31	33.67	32.84	29	19.44
01033	0	0	0	0	0	0	0
0104	0	0	0	0	0	0	0
References				[Steffense et al., 1998]	[Zhao et al., 2001]	[Grimsley et al., 2002]	[Matte C et al., 2000]

[a] compared with Danish $\chi^2 = 15.64$, $p = 0.0004$.

[b] compared with Danish $\chi^2 = 24.58$, $p = 0.0000046$.

[c] compared with Danish $\chi^2 = 14.69$, $p = 0.00065$.

[b] compared with Indigenous African $\chi^2 = 7.61$, $p = 0.022$.

CHAPTER VI

DISCUSSION

In the present study, polymorphism within the HLA-E gene could be found at codon 77 and 107 whereas none were detected at codon 2, 82, 83, 150 and 157. These finding were not surprising because several studies reported the absence of polymorphism at these positions as well (Steffense et al., 1998; Matte et al., 2000; Zhao et al., 2001; Grimsley et al., 2002). The polymorphism at codon 77 is a silent mutation, whereas polymorphism at codon 107 leads to a missense mutation. This mutation displays an amino acid alteration from arginine to glycine. The physical distance between SNP at codon 77 and 107 was approximately 329 nucleotides.

In this study, we used population-based case-control study to investigate association of a marker allele with NPC by comparing the frequency of the allele in a disease population with that in a disease-free population (Allahabadia and Gough, 1999). Analysis of SNP at codon 107 showed a significant association between HLA-E107G allele and NPC in Thai population, suggesting the role of HLA-E as an NPC susceptibility gene. However, this association is not statistically significant in Chinese and Thai-Chinese population. There are a number of possible explanations for the association between HLA-E and NPC. First, the lack of association of this allele in Chinese and Thai-Chinese population does not support the importance of HLA-E as NPC susceptibility gene because NPC is most common in Chinese (Liebowitz, 1994). However, it is possible that the sample size of Chinese and Thai-Chinese of NPC patients in this study was not enough for statistical analysis. Our finding need to be studied in a larger population to investigate the association between HLA-E polymorphism in Chinese and Thai-Chinese patients with NPC. In addition, the definition for ethnicity in this study is self-reporting which might be inaccurate. In summary, the analysis of all the samples together as Thai/Chinese indicates the role of HLA-E107G SNP in NPC development. Second, there may be some other genes in this region that

play a role in the susceptibility to NPC that are in linkage disequilibrium with HLA-E107G (Tabor et al., 2002). However, in this study we also performed haplotype analysis to further elucidates the relevance of the two SNPs and NPC. No significant risk of the haplotype associated with NPC has been detected in all groups. This data of haplotype analysis supported the importance of HLA-E107G SNP, which contributed to low risk in NPC development. However, linkage disequilibrium analysis by spanning at least 30-50 kb is required to answer the hypothesis whether another linked gene is responsible for NPC development (Jorde, 2000). Third, these observations might confer false-positive results. Although case-control study is a sensitive method and is more likely to detect genes of the modest effect, this strategy is prone to inconsistent results due to false positive that may arise from population stratification, which are also more likely to occur in small data set. This problem can be overcome by using family-based association study (Allahabadia and Gough, 1999).

If HLA-E107G is a true disease allele, which is likely one of several causes for NPC development. Various explanations that can link HLA-E to NPC development were discussed below.

Firstly, HLA-E plays an important role in NK cell function. HLA-E has been found to present class I leader peptides and to be recognized by natural killer cells (Lee and Llano et al., 1998; Lee and Goodlett et al., 1998). This recognition is mediated by the interaction of HLA-E with the CD94/NKG2 receptor and can result in either inhibition or activation of the natural killer cell, depending on the peptide presented and which NKG2 receptor it associates with (Lee and Llano et al., 1998; Braud et al., 1998; Llano et al., 1998). One experiment supporting function of HLA-E107G to present the leader peptide of certain HLA-A, -B, -C and –G alleles to NK cell receptors and affect different NK cell function. HLA-E presents the leader peptide from A2, which is common type in most population and there are reports that HLA-A2 was associated with NPC (Simons et al., 1974; Chan et al., 1983). So, it is possible that HLA-E107A presents the leader peptide from HLA-A2 leading to no inhibition in NK cell-mediated lysis. Whereas HLA-E107G allele with the leader peptide from HLA-A2 will inhibit NK cell-mediated lysis

(Maier S et al., 2000). It would be interesting to further characterize HLA-A together with HLA-E allele in NPC patients. In addition, One study showed the relation between NPC and NK cell that NK cell activity of NPC patients was lower significantly than controls (Lynn et al., 1986). This might be the effect of HLA-E107G allele to inhibit NK cell-mediated lysis.

Secondly, according to the crystal structure of HLA-E (O'Callanghan and Tormo et al., 1998), the amino acid at position 107 is found in a loop between the β-pleats at the base of the antigen binding cleft in the α -2 domain. This position is not placed in the peptide-binding region but rather in the T cell receptor-contacting region. Recent studies (Martinazzi et al., 1999) demonstrated that HLA-E complexed with class I signal sequence-derived peptides is not only a ligand for NK cell inhibitory receptors, but can also interact with TCR and trigger CTL (Martinazii et al., 1999). Furthermore, HLA-E can also bind peptides derived from viral proteins such as EBV peptide (BZLF-1 $_{39\text{-}47\ \text{nonamers}}$) (Ulbrecht et al., 1998) and can interact with αβ TCRs expression $CD8^+$ T cells to trigger CTL function (Garcia et al., 2002). Interestingly, this virus has been shown the strong association with NPC (Henle W and Henle G, 1979) and EBV latent gene products EBERs, EBNA-1, LMP-1 and LMP-2 were detected in the tumor cells (Rickinson AB and Kieff, 1996). Yet, no direct proof for functional implication of CTL response in the interaction between TCR and each HLA-E allele bind to EBV peptides. However, the study of Garcia et al. (2002) reported the facts that K14 (CTL clone) efficiently reacted with HLA-E in both experimental systems indicated that it does not discriminate between both types of alleles (Garcia et al., 2002).

Several observations raise the possibility that HLA-E might plays an important role in the regulation of CTL function, as CD94/NKG2 receptors are also expressed on T cells in particular on those with an activated or memory phenotype (Braud et al., 1999; McMahon and Raulet, 2001). The functional role of HLA-E-NK cell receptor recognition on CTLs remains unclear. One study of Malmberg et al. (2002) demonstrated that CD94/NKG2 receptors play an important role in protecting tumors from CTL-mediated lysis via the upregulation of HLA-E (Malmberg et al., 2002). This might be a tumor

escape mechanism for CTL-mediated immunity. However, future studies would be interesting to test the functional properties of the HLA-E107A/G alleles in NK cell or CTL for NPC patients.

In addition, our study showed the basic knowledge of the diversity of HLA-E in Thailand. We observed the presence of 3 out of 6 previously characterized HLA-E alleles, HLA-E*0101, *01031 and *01032. This finding was similar to previously reports in African-American, Japanese, Danish, Shanghai Chinese and indigenous African (Steffense et al., 1998; Matte et al., 2000; Zhao et al., 2001; Grimsley et al., 2002). In the present study, the data of HLA-E allele frequencies reveal the limited polymorphism of HLA-E with some difference patterns in the distribution of alleles in ethnically non-related populations.

CHAPTER VII

CONCLUSION

The aim of this research was to explore a risk association between SNPs of the HLA-E gene and NPC. The results indicated that HLA-E107G allele might be important in the nasopharyngeal carcinogenesis in the Thai population. The risk association was detected for homozygous HLA-E107G with a significant OR (95% CI) of 2.11(1.15-3.88) and p=0.009. Although the genetic evidence remains to be further proved, various possibilities about the role of HLA-E in NPC development can be hypothesized. One possibility is that HLA-E107G presents certain HLA class I-derived or EBV peptide may inhibit NK cell-mediated tumor lysis. Another mechanism leading to NPC development is that HLA-E107G combined with HLA class I-derived or EBV peptide may lead to enhance inhibitory signal to CTLs via CD94/NKG2 receptors and thereby turns off their effected function. This may constitute a tumor escape mechanism from CTL-mediated immunity. In addition, HLA-E107G allele might affects the efficiency of HLA-E bound to EBV peptide and therefore cannot stimulate CTL via TCR to eliminate tumor cells or viral infected cells.

Moreover, this study reveals the HLA-E polymorphism in Thailand, which comprised Thai, Chinese and Thai-Chinese population. These populations contain low levels of allelic polymorphism similar to ethnic groups from other countries. However, different pattern of allele distributions was found when compared to Caucasian and African populations.

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APPENDICES

APPENDIX I

Reagent for agarose gel electrophoresis

1. 50x Tris-acetate buffer (TAE)

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

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

2. 10mg/ml Ethidium bromide

Ethidium bromode	1	g
Distilled water	100	ml

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

3. 1.5% Agarose gel

Agarose	0.3	g
1x TAE	20	ml

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

APPENDIX II

Reagent for dot blot hybridization

1. 20x SSC

3 M NaCl	175.3	g
0.3 M Sodium citrate	88.2	g
Distilled water	800	ml

Dissolved these components and adjust pH to 7.0 with NaOH (6.5 ml of a 10 N solution). Adjust volume to 1 liter. Dispense into aliquots. Sterilize by autoclaving.

2. Denaturing solution

1.5 M Sodium chloride	87.75	g
0.5 M sodium hydroxide	20.0	g
Distilled water	800	ml

Dissolved these components and adjust volume to 1 liter.

3. Neutralizing solution

1.5 M Sodium chloride	87.66	g
0.5 M Tris –HCl pH 7.2	60.57	g
0.5 M EDTA pH 8.0	2	ml
Distilled water	800	ml

Dissolved these components and adjust volume to 1 liter.

4. Hybridization solution (per 1 reaction)

20x SSC	5	ml
10% Blocking agent	2	ml

10% N-lauroylsarcosine	2	ml
10% SDS	40	µl

Adjust volume to 20 ml with distilled water.

5. **Washing solution**

20x SSC	250	ml

Make up to 1 liter.

APPENDIX III

Estimating Haplotype (EH)

File in this Window package

EH.PAS : Source code of EH program.

EH.EXE : Expectable code of EH program, which is compiled with a maximum of 30 alleles per locus, 10 loci, 1,000 haplotypes, and 3,600 genotypes (product of numbers of genotypes at each locus).

EH.DAT, EH.OUT : Sample input and output files.

Protocol for estimating haplotype frequencies

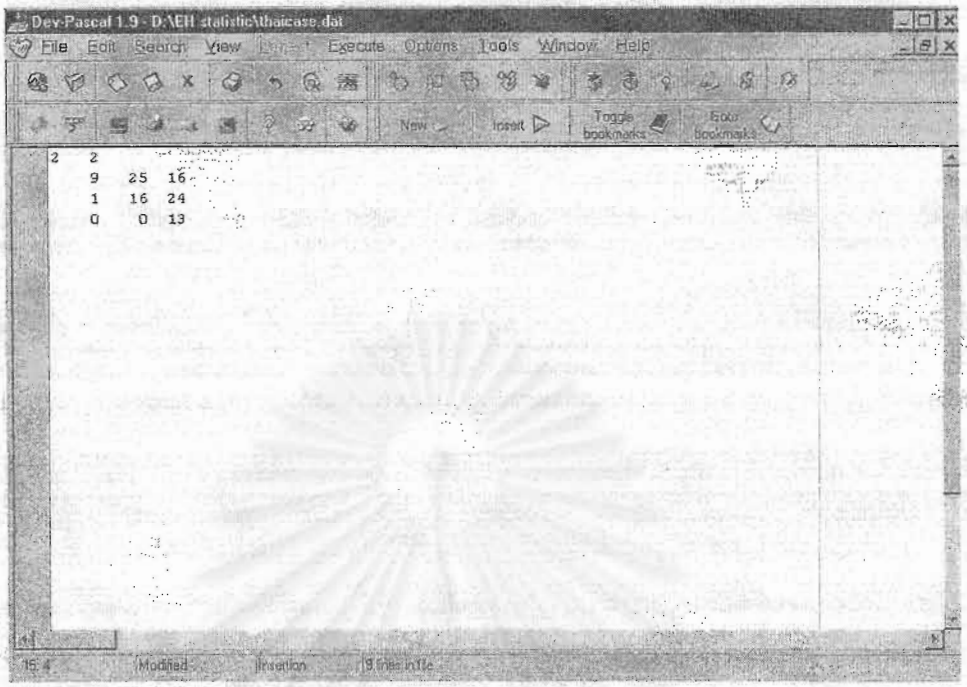
1. Create the data file (.dat)

We created the data file in the Pascal program. The first line was the number of alleles at the first locus, number of alleles at the second locus, and so on. Assuming we have 2 loci, each locus has 2 alleles, we write 2 2 in the first line. The subsequent lines were the numbers of the possible haplotype. For example, we created the data file from Thai case in this study. The number of haplotype must be arranged as follow:

		Second locus	
	AA	AG	GG
CC	9	25	16
CT	1	16	24
TT	0	0	13

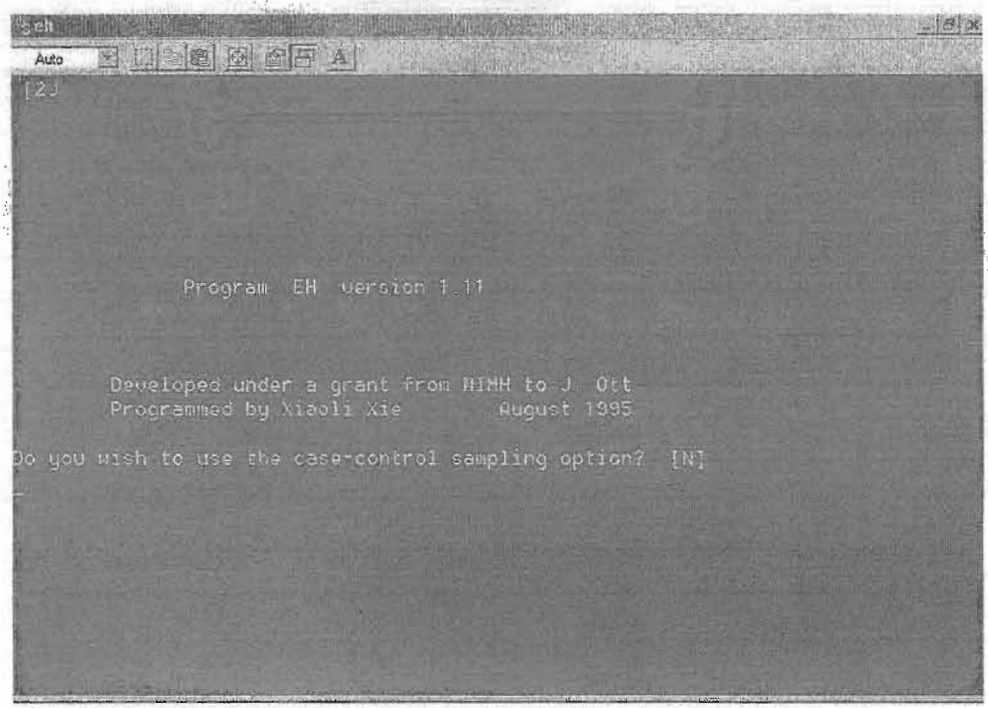
First locus

The number of haplotype was filled in Pascal program and save unit as thaicase .dat file. The window of Pascal program was showed below.

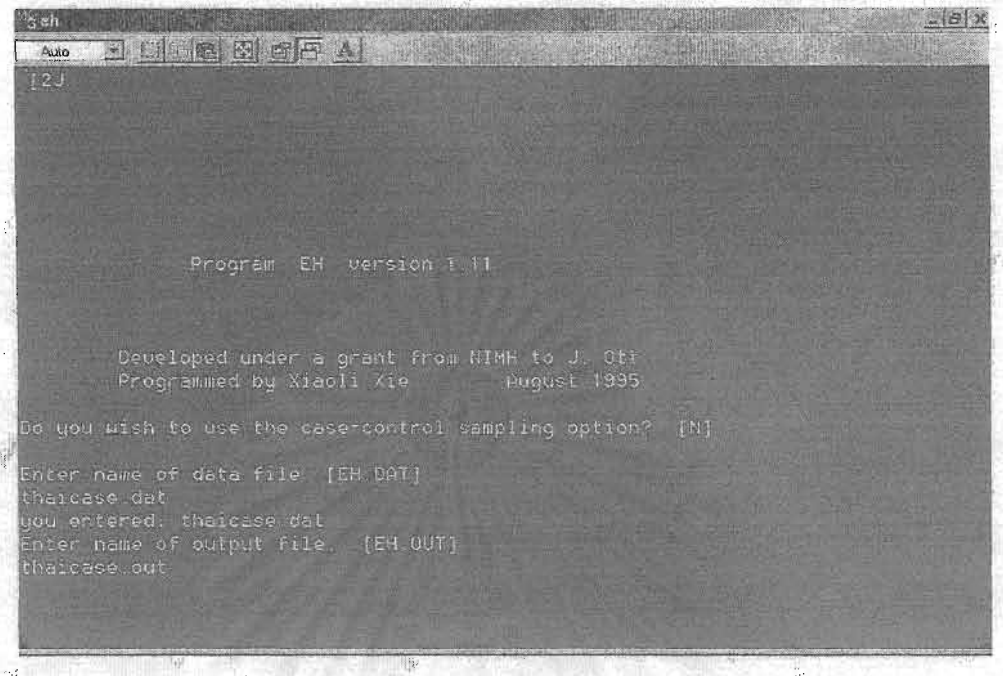


2. Running the EH program

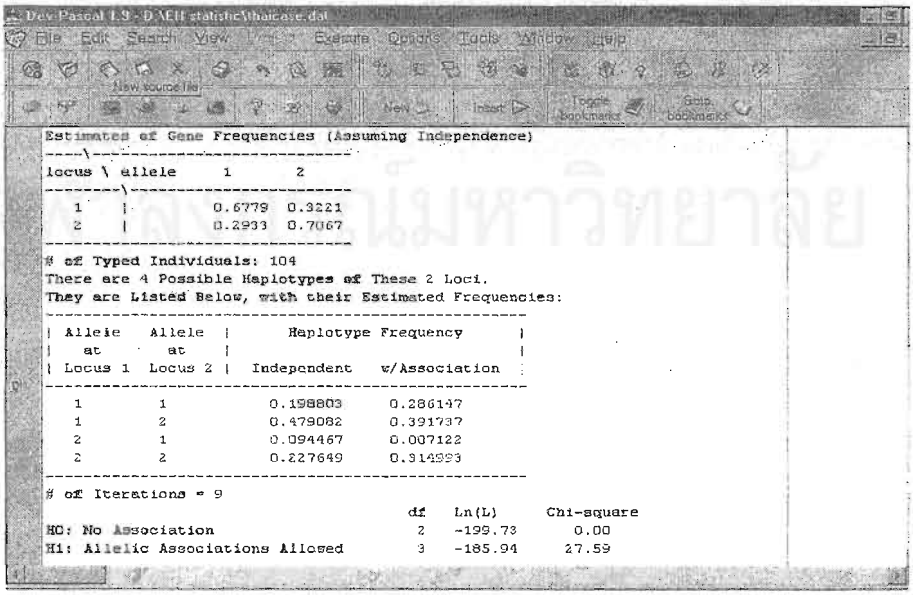
- Running EH program showed the window as below.



- Type data filename (thaicase.dat) and output filename (thaicase.out).



- The output file presented the haplotype frequencies in two kind. "Independent" these are obtained from the allele frequencies at the individual loci. That is, these haplotype frequencies are not estimated but calculated from allele frequencies under the assumption of no association. "w/association" these are estimated from the data, allowing for association (linkage disequilibrium), assuming Hardy Weinberg equilibrium. Our study used haplotype frequencies data from w/association.



Case-control data

To test whether haplotype frequencies are significantly different in case and controls, we run EH three times, 1) for cases, 2) for controls, and 3) for cases and controls combined. For a given data set (case.dat, control.dat, provided). The representatives of results are shown below.

Thaicase.out

	df	Ln(L)	Chi-square
HO: No Association	2	-199.73	0.00
H1: Allelic Associations Allowed	3	-185.94	27.59

Thaicontrol.out

	df	Ln(L)	Chi-square
HO: No Association	2	-189.50	0.00
H1: Allelic Associations Allowed	3	-171.85	35.31

Thaicase+Thaicontrol.out

	df	Ln(L)	Chi-square
HO: No Association	2	-394.59	0.00
H1: Allelic Associations Allowed	3	-362.58	64.02

The relevant test statistic is T (χ^2) = ln (L,cases) + ln (L,controls) – ln (L,cases + controls together). With a sufficient number of observation, when there is no difference between case and control haplotype frequencies, twice this value has an approximate chi-square distribution with a number of df equal to the number of haplotype estimated. For the above data, one obtains (-185.94)+(-171.85)-(-362.58) = 4.79 χ^2 = 2 * 4.79 = 9.58 on 3 df is associated with an empirical significance level of <0.025. In addition, this formula can also used to test the relevant difference of cases and cases or controls and controls in different ethnic groups.

Table: Chi-square Probabilities

The areas given across the top are areas to the right of the critical value. To look up an area on the left, subtract it from one and then looks it up (ie: 0.05 on the left is 0.95 on the right).

df	0.995	0.99	0.975	0.95	0.90	0.10	0.05	0.025	0.01	0.005
1	---	---	0.001	0.004	0.016	2.706	3.841	5.024	6.635	7.879
2	0.010	0.020	0.051	0.103	0.211	4.605	5.991	7.378	9.210	10.597
3	0.072	0.115	0.216	0.352	0.584	6.251	7.815	9.348	11.345	12.838
4	0.207	0.297	0.484	0.711	1.064	7.779	9.488	11.143	13.277	14.860
5	0.412	0.554	0.831	1.145	1.610	9.236	11.070	12.833	15.086	16.750
6	0.676	0.872	1.237	1.635	2.204	10.645	12.952	14.449	16.812	18.548
7	0.989	1.239	1.690	2.167	2.833	12.017	14.067	16.013	18.475	20.278
8	1.344	1.646	2.180	2.733	3.490	13.362	15.507	17.535	20.090	21.955
9	1.735	2.088	2.700	3.325	4.168	14.684	16.919	19.023	21.666	23.589
10	2.156	2.558	3.247	3.940	4.865	15.987	18.307	20.483	23.209	25.188

BIOGRAPHY

Miss Ingorn Kimkong was born on December 10, 1978 in Ratchaburi, Thailand. She graduated with the Bachelor degree of Science in Medical Technology from Thammasat University in 2000 and then attended to particulate in Medical Microbiology program, Graduate School, Chulalongkorn University for her master degree.