รายงานวิจัยฉบับสมบูรณ์

โครงการ: การค้นหา cytotoxic T lymphocyte epitopes ของ latent membrane protein 1 จาก Epstein-Barr virus ที่พบในมะเร็งหลังโพรงจมูกในประชากรไทย

โดย

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ชื่อโครงการวิจัย: การค้นหา cytotoxic T lymphocyte ของ latent membrane protein 1 จากไวรัสเอปสไตน์บาร์ที่พบในมะเร็งหลังโพรงจมูกในประชากรไทย

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การกระตุ้นภูมิคุ้มกันเชิงเซลล์ชนิด cytotoxic T lymphocyte ที่จำเพาะต่อโปรตีนของไวรัสเอปสไตน์บาร์ที่สัมพันธ์กับมะเร็งหลังโพรงจมูกเป็นแนวทางหนึ่งในการพัฒนาแนวทางการรักษาต่อโรคมะเร็งหลังโพรงจมูก การศึกษานี้เป็นขั้นตอนหนึ่งที่จะนำไปสู่การพัฒนาการรักษานั้น เริ่มด้วยการศึกษารูปแบบของ HLA class I ของผู้ป่วยโรคมะเร็งหลังโพรงจมูกในประชากรไทย เนื่องจากข้อมูลเกี่ยวกับรูปแบบของ HLA class I ชนิดที่พบได้บ่อยมีประโยชน์ในขบวนการค้นหาส่วนของเปบไทด์ที่สามารถกระตุ้น cytotoxic T lymphocyte ได้ ชนิดของ HLA ที่พบได้บ่อยมากกว่า 10% ในประชากรไทย ได้แก่ A2 (58%), A11 (43%), A19 (A33) (32%), A9 (A24) (26%), B46 (30-46%), B40 (B60) (19-24%), B58 (12-24%), B12 (B44) (9-11%), and B75 (11%) นอกจากนั้นการศึกษานี้ยังรวมถึงการหาลำดับเบสของ LMP1 โดยใช้ตัวอย่างชิ้นเนื้อของมะเร็งหลังโพรงจมูกจากประชากรไทย โดยเน้นที่บริเวณที่เป็น CTL epitopes ที่มีผู้ศึกษาไว้แล้วเพื่อดูระดับของความหลากหลายในบริเวณนั้น โดยสรุป CTL epitopes 2 ชนิดที่จำเพาะกับ HLA A2 (ALLVLYSFA and LLLIALWNL) และ อีก 2 ชนิดที่จำเพาะกับ HLA B31/33 และ B61 (SDSNSNEGR and NEGRHHLLV) จากไวรัสเอปสไตน์บาร์ในประชากรไทยมีลำดับเบสที่มีความเหมือนกันกับข้อมูลเดิมจากประชากรอื่นๆค่อนข้างสูง อย่างไรก็ตามผลของไวรัสเอปสไตน์บาร์จากชิ้นเนื้อมะเร็งหลังโพรงจมูกจากประชากรไทยแสดงถึงการเปลี่ยนแปลงที่ทำให้เกิดการเปลี่ยนชนิดของกรดอะมิโนในบางตำแหน่งด้วย ส่วน CTL epitope อีก 1 ชนิดที่จำเพาะกับ HLA A2 (YLLEMLWRL) นั้นมีลำดับเบสคล้ายคลึงกับข้อมูลจากประเทศจีน (YFLEILWRL) การศึกษาสุดท้ายคือการค้นหาส่วนของเปบไทด์ที่สามารถกระตุ้น cytotoxic T lymphocyte จากโปรตีนของไวรัสเอปสไตน์บาร์ที่สัมพันธ์กับมะเร็งหลังโพรงจมูกที่จำเพาะกับ HLA class I ชนิดที่พบได้บ่อยในประชากรไทยตรวจโดยใช้ชุดของ overlapping peptide ในตัวอย่างคนไทย 20 ราย ผลการตรวจพบว่ามีเพียง 2 ใน 20 รายที่แสดงผลบวกต่อเปบไทด์ขนาดยาว 1 ชนิด (SNSNEGRHHLLVSGAGDD) อย่างไรก็ตามผลนี้ต้องการการศึกษาเพิ่มเติมเพื่อค้นหาเปบไทด์ขนาดสั้นภายในนั้นรวมถึงการบ่งชี้ชนิดของ HLA ที่จำเพาะต่อเปบไทด์นี้ต่อไป

คำหลัก: NPC, EBV, CTL epitope, LMP1, HLA

ABSTRACT

Project Code: PDF/25/2544

Project Title: Mapping of cytotoxic T lymphocyte epitopes within latent membrane protein 1 from nasopharyngeal carcinoma-associated Epstein-barr virus in Thai population

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To develop a therapeutic tool for the treatment of NPC by the delivery of a curative EBV-specific immune response to NPC patients, one approach is to stimulate the CTL against EBV antigen present in the tumor cells. As a first step toward that goal, this study characterized HLA class I distribution from Thai NPC patients since the HLA information particularly the one common in the endemic area is very useful for the characterization process of CTL epitopes. The common HLA antigens with antigen frequencies (AgF) of more than 10% in Thai NPC were A2 (58%), A11 (43%), A19 (A33) (32%), A9 (A24) (26%), B46 (30-46%), B40 (B60) (19-24%), B58 (12-24%), B12 (B44) (9-11%), and B75 (11%). In addition, this study characterized LMP1 sequence from Thai NPC biopsy, particular focusing on the region containing previously identified LMP1 epitopes to assess their degree of variability. In summary, the 2 HLA A2-restricted (ALLVLYSFA and LLLIALWNL) and HLA B31/33 & B61-restricted (SDSNSNEGR and NEGRHHLLV) epitopes were highly conserved in virus isolates from Thai NPC biopsy compared to available data. However, data from Thai NPC isolates revealed unique sequence which resulted in amino acid substitution. As for the more diverse HLA A2-restricted epitope, YLLEMLWRL, Thai isolates had similar sequences as other isolates from Chinese (YFLEILWRL). Lastly, the CTL epitopes within NPC-associated LMP1 antigen restricted through common HLA class I alleles prevalent in Thai population were mapped by screening overlapping peptides in 20 donors. Only 2 out of 20 donors gave positive responses to a long peptide (SNSNEGRHHLLVSGAGDD containing a potential novel epitope). However, the minimal epitope and HLA-restriction of the epitope still need further characterization.

Keywords NPC, EBV, CTL epitope, LMP1, HLA

EXECUTIVE SUMMARY

NPC is one of the most common forms of cancer in some regions of China and South-East Asia and occurs less commonly in North Africa. Although patients with early stage of the disease are highly curable by radiotherapy, about 10% of the patients might not respond to the treatment. In addition, patients with advanced lesions are poorly controlled by radiation and recurrences are usually occurred within 5 years leading to only 10-40% five year survival rate. Since NPC is strongly associated with EBV infection, the stimulation of the patient's immune responses against the viral antigens might provide a useful tool of rejecting these virus associated tumor cells. To develop a therapeutic tool for the treatment of NPC by the delivery of a curative EBV-specific immune response to NPC patients, one approach is to stimulate the CTL against EBV antigen present in the tumor cells. As a first step toward that goal, this study characterized HLA class I distribution from Thai NPC patients compared to normal healthy control since the HLA information particularly the one common in the endemic area is very useful for the characterization process of CTL epitopes. The common HLA antigens with antigen frequencies (AgF) of more than 10% in Thai NPC were A2 (58%), A11 (43%), A19 (A33) (32%), A9 (A24) (26%), B46 (30-46%), B40 (B60) (19-24%), B58 (12-24%), B12 (B44) (9-11%), and B75 (11%). In addition, this study characterized LMP1 sequence from Thai NPC biopsy, particular focused on the region containing previously identified LMP1 epitopes. The HLA A2-restricted (ALLVLYSFA and LLLIALWNL) and HLA B31/33 & B61-restricted (SDSNSNEGR and NEGRHHLLV) epitopes were highly conserved in virus isolates from Thai NPC biopsy compared to available data. However, data from Thai NPC isolates revealed unique sequence which resulted in amino acid substitution. As for the HLA A2-restricted epitope, YLLEMLWRL, Thai isolates had similar sequences as other isolates from Chinese (YFLEILWRL). Lastly, the CTL epitopes within NPC-associated LMP1 antigen restricted through common HLA class I alleles prevalent in Thai population were mapped by screening overlapping peptides in 20 donors. Only 2 out of 20 gave positive responses to a 18-mer peptide (SNSNEGRHHLLVSGAGDD). However, the minimal epitope and HLA-restriction of the epitope still need further characterization. Since this protein is poorly immunogeneic, to target EBV-positive NPC tumor, such small response against LMP1 should be amplified. Therefore, more donors should be included to obtain more putative CTL epitopes of LMP1.

LIST OF SYMBOLS

NPC	NASOPHARYNGEAL CARCINOMA
EBV	EPSTEIN BARR VIRUS
LMP	LATENT MEMBRANE PROTEIN
EBNA	EPSTEIN BARR VIRUS NUCLEAR ANTIGEN
HLA	HUMAN LEUKOCYTE ANTIGEN
CTL	CYTOTOXIC T LYMPHOCYTE
IFN-□	INTERFERON GAMMA
PTLD	POST TRANSPLANT LYMPHOPROLIFERATIVE DISEASE
PBMC	PERIPHERAL BLOOD MONONUCLEAR CELL
PCR	POLEMERASE CHAIN REACTION

INTRODUCTION

NPC is one of the most common forms of cancer in some regions of China and South-East Asia and occurs less commonly in North Africa. Although patients with early stage of the disease are highly curable by radiotherapy, about 10% of the patients might not respond to the treatment. In addition, patients with advanced lesions are poorly controlled by radiation and recurrences are usually occurred within 5 years leading to only 10-40% five year survival rate. Since NPC is strongly associated with EBV infection, the stimulation of the patient's immune responses against the viral antigens might provide a useful tool of rejecting these virus associated tumor cells. The link between EBV and the geographically constrained NPC is not entirely clear but is believed to be influenced by dietary and genetic factors. It is now well established that these tumor cells, like many other EBV-associated malignancies, express a limited number of EBV antigens thus restricting the potential targets for immune recognition (reviewed in Khanna and Burrows, 2000). Many laboratories were studying the immune mechanisms responsible for protecting healthy individuals from a recrudescence of infection with EBV. They have shown that the main immunological arm responsible for this protection involves cytotoxic T lymphocytes (CTL) although these T cells fail to control the outgrowth of some EBV-associated malignancies (reviewed in Khanna et. al. 1995; reviewed in Khanna *et. al.* 1999a; reviewed in Rickinson & Moss 1997).

In the case of NPC, the precise mechanism of immune escape is unclear. Earlier studies have suggested that most NPC patients retain detectable EBV-specific T cell surveillance, indicating that CTL dysfunction is an unlikely cause of the outgrowth of these tumors *in vivo* (Moss *et. al* 1983). Based on studies in healthy individuals, it is likely that this response is strongly focussed through epitopes within the EBV nuclear antigens (EBNA) 2-6 proteins with minimal HLA class I-restricted reactivity against latent membrane proteins (LMP1 & LMP2) and none within EBNA1 (Levitskaya *et al*, 1995). Since NPC tumor cells express only EBNA1 and LMP1 & 2, the existing EBV-specific CTL repertoire in NPC patients may have a limited capacity to control this tumor *in vivo*. The lack of class I-restricted processing of EBNA1 has directed an increasing interest in designing strategies to enhance the response to LMP epitopes to control NPC. Before such strategies can be designed it is important to precisely map the CTL responses to

EBV antigens expressed in NPC. Previously attempts to map such responses have been constrained by the ability to expand virus-specific CTLs *in vitro* to numbers which allow functional analysis in cytotoxicity assays. A major advancement in screening protocols has come with development of a rapid single cell assay which is based on the peptide induced secretion of interferon γ (IFN-γ) by T cells (also refered to as ELISPOT assay). This assay allows a rapid means of profiling epitope-specific CTL responses without long-term *in vitro* manipulation.

Currently, most of the defined CTL epitopes from LMP were characterized from the Caucasian healthy donors (reviewed in Khanna et al., 1999a, donors (reviewed in Khanna and Burrow, 2000). Since it has recently been shown that the EBV strains in China and Southeast Asia NPC patients posses a unique LMP sequences (Hu et al., 1991; Miller et al., 1994; Busson et al., 1995; Walling et al., 1999; khanna et al., 1997a); therefore, new epitopes identified from EBV strain found in these population is also required. Moreover, it is necessary to identify additional epitopes restricted to common HLA type in Southeast Asia population.

The overall long-term aim of this project is to develop a rational basis for the delivery of a curative EBV-specific immune response to NPC patients. One approach is to stimulate the CTL against EBV antigen present in the tumor cells by immunizing protocol. It is anticipated that these T cells subsequent to the completion of this project will be used as a therapeutic tool for the treatment of NPC by analogy to that used for other EBV-associated cancers (reviewed in Rooney et al., 1998a; reviewed in Heslop et al., 1997). As a first step toward our goal, this study proposes to 1) characterize HLA class I distribution from Thai NPC patients compared to normal healthy control, 2) characterize LMP1 sequence from Thai NPC patients compared to available data, and 3) map the CTL epitopes within NPC-associated LMP1 antigen restricted through common HLA class I alleles prevalent in Thai population.

PROCEDURE

Experimental design

This study proposed to characterize LMP1 sequence from EBV strain commonly found in Thai NPC patients and compared with the available data to synthesize a set of overlapping peptide to use for the epitope mapping in Thai population. The donor will be selected from healthy carriers who carry common HLA class I alleles prevalent in Thai population as shown in table 6. Overlapping peptides will be prepared and tested. In brief, the LMP1 protein contains 386 amino acids (Kieff, 1996), the total of forty-five overlapping peptides were synthesized and tested in this study.

Methods

Study Population for HLA study.

The study population consists of (1) newly diagnosed NPC patient, (2) long term survival patient, and (3) EBV-positive healthy donors in Thailand. Specifically, we recruited the patients who attend King Memorial Chulalongkorn Hospital that have been diagnosed as undifferentiated NPC by accepted histopathological criteria. The long term survival group is the NPC patients who had been previously treated with radiotherapy or combined radio-chemotherapy and were free of clinical disease at the time of the study for more than 1 year. The healthy carrier individuals of Thai origin were recruited from donors at the Thai Red Cross Society. After obtaining an informed consent, peripheral blood samples were taken from healthy EBV-positive donors and NPC patients. Blood were fractionated on Ficoll density gradients, and PBMC were collected to be used in HLA serological typing and the ELISPOT assay. In addition, DNA were prepared from peripheral blood samples and from serum using Qiamp DNA blood mini kit (Qiagen, Basel Switzerland) according to the "blood and body fluid protocol".

Study population for CTL mapping.

After obtaining informed consent, healthy virus carrier with required-HLA class I allele, and is homozygous on that locus if possible, were recruited as donor in this study. Approximately 20 donors were included in the CTL screening process.

Determination of HLA Alleles.

HLA-A and HLA-B alleles were determined using standard microcytotoxicity test, which is a methodology routinely performed in Immunology unit, Chulalongkorn University. Briefly, lymphocyte separation and HLA typing were performed from freshly drawn blood by standard microlymphocytotoxicity assay. The panel of 70 antisera were used to defined the 17 HLA-A antigens and 30 HLA-B antigens. The antigen frequencies were determined by direct counting.

Certain HLA alleles such as HLA-A2 subtype were determined using the DNA-based Sequence Specific Primer (SSP) typing procedure (Krausa et al., 1995). In addition, the high resolution typing of HLA-B locus were done by direct sequencing of the PCR products using primers BIN1-TA, BIN1-CG and BIN3, which amplified exons 2 and 3 and intron 2 as previously described (Pimtanothai et al., 2000).

LMP Sequencing.

LMP1 gene were amplified from genomic DNA prepared from NPC tumor biopsy using LMP1-specific primers. Then the PCR were purified and subjected to direct sequencing using automated sequencer (310 Genetic Analyzer). Samples randomly selected from a pool of ~ 200 samples of NPC tumor DNA have been previously prepared and will be kindly given to us by Dr. Mutirangura.

DNA sequencing.

For direct cycle sequencing, 40 µl of the PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN Inc.) to obtain clean double-standed DNA amplificates. Cycle sequencing was performed on an ABI Prism 310 Genetic Analyzer using a cycle sequencing chemistry with base-specific fluorescence – labeled dideoxynucleotide termination reagents, BigDye Terminator Ready Reaction Mix (Applied Biosystems) was used for sequencing. Thus, each sequencing reaction mixture of 10 µl final volume contained 1 µl of 5 pmol primer, 3 µl of template and 3 µl of the BigDye Terminator Ready Reaction Mix. Each sample mixture was then subjected to a cycle sequencing reaction in a Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The condition of cycle sequencing reaction consisting of denaturation at 96° c for 30 seconds, annealing at 55° c for 10 seconds and extension at 60° c for 4 minutes were carried out. Then each sequencing reaction product was pooled into 2 µl of 3 M sodium acetate (NaOAc), pH4.6/50 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.

Overlapping peptides.

In the screening process, segments of 20 amino acids, overlapping by 10 amino acids and spanning the entire molecule based on the sequencing data were synthesized by Chiron Technologies using the solid phase method. In addition, segments of 9 amino acids, overlapping by 1 amino acid, spanning the region of the 18-20-mers that gives positive result in the ELISPOT assay will be synthesized by the same method. One 18-20-mers peptide was composed of twelve 9-mers peptides. The rational for this design is because the usual length of CTL epitope is ~9-10 aa long. Peptides were dissolved in DMSO, and diluted in serum-free RPMI 1640 medium for use in the ELISPOT assay.

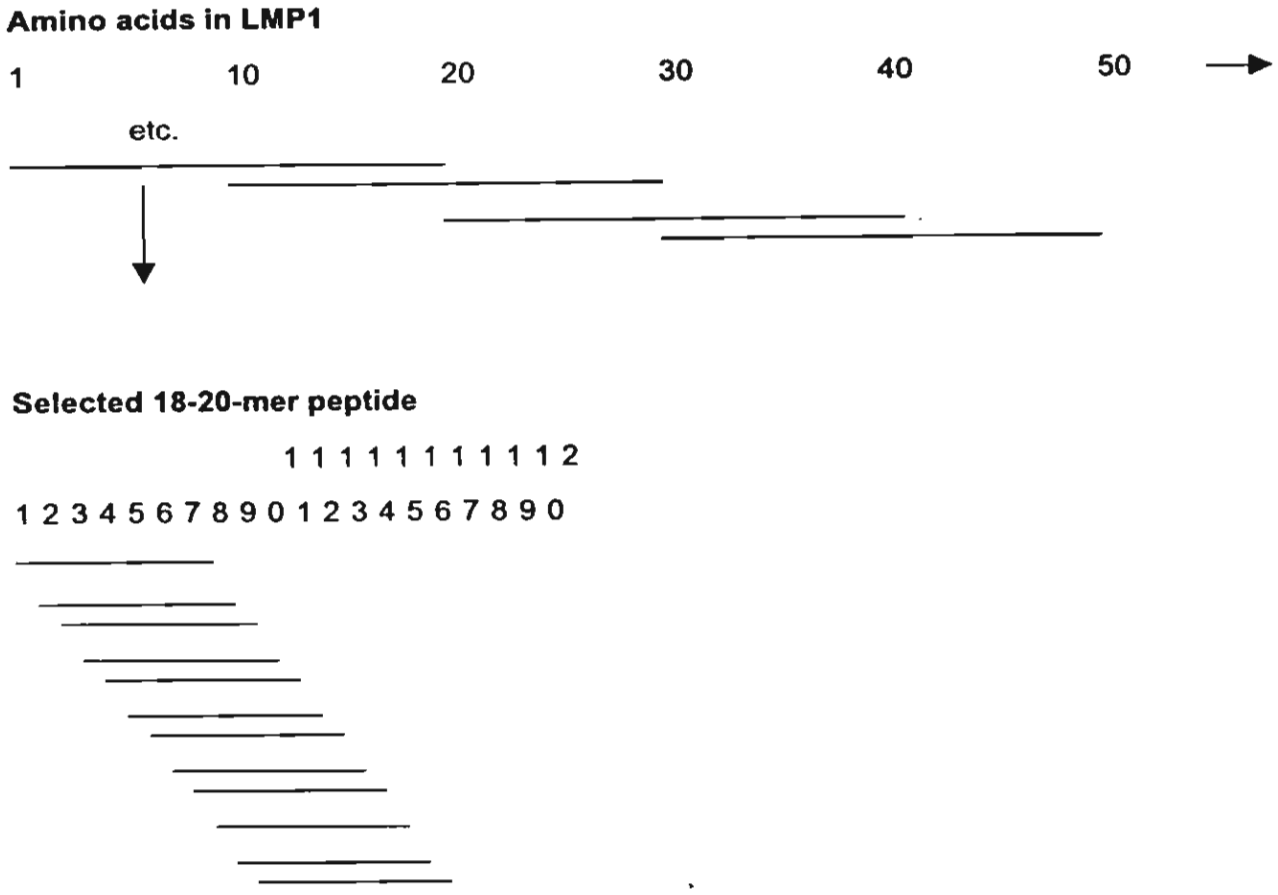

Figure 1. a diagram shows an example of the overlapping segments

ELISPOT assay for single cell IFN-γ release.

Nitrocellulose membrane-base 96 well plates were precoated with anti-IFN-—γ monoclonal antibody. PBMC (250,000 cells/well) were added in the presence of each peptide at a predetermined optimal concentration (~5-10μg/ml) in triplicate and incubated overnight. The cells were discarded the following day and incubated with biotinylated anti-IFN-—γ monoclonal antibody, followed by strepavidin-conjugated alkaline phosphatase. Individual IFN-γ producing cells were detected as dark spots after adding the substrate and can be counted using dissection microscope.

RESULTS

The distribution of HLA-A and HLA-B in NPC patients compared to normal control

The study population included 53 unrelated Thai patients with histologically confirmed NPC diagnosed at King Chulalongkorn Memorial Hospital in Bangkok. There were 34 men and 20 women, with a median age of 48 years (range, 16-81). All of these patients were positive for the EBNA1 gene in tumor cells as detected by PCR as reported previously (Mutirangura et al., 1997 and 1998). Seventy healthy unrelated Thai individuals served as ethnically and geographically matched controls.

The distribution of HLA-A and -B antigens between the two groups is shown in Table 1 and 2. A total of 10 HLA-A antigens and 23 HLA-B antigens were observed in the Thai control group. The common HLA antigens with antigen frequencies (AgF) of more than 20% in Thai controls were A2 (38.5%), A9 (A24) (42.8%), A11 (48.6%), A19 (A33) (34.3%), B12 (B44) (20%), B13 (22.8%), and B40 (B60) (22.8%). Ten HLA-A antigens and 21 HLA-B antigens were detected in NPC patients with the same 4 common HLA-A antigens observed at high frequencies (26.4-58.5%). However, the common HLA-B antigens in NPC patients were different from the control group. B17 (B58) and B46 antigens were present in the patient group with the high AgF of 24.5% and 30.2%, respectively.

The HLA-B antigens are highly polymorphic with more than 100 alleles described. In addition, many HLA-B alleles have been previously described as an important associated factor with NPC. Therefore, we have also performed a high resolution typing of HLA-B by direct sequencing method in a subset of sample (54 NPC patients compared to 49 controls). The distribution of HLA-B alleles between the two groups is shown in Table 3. A total of thirty-four HLA-B alleles was observed in the Thai control group including one new allele (B*3804) (Steiner et al., 2001). The five most common alleles in Thai controls were B*44032 (28.5%), B*4601 (16.3%), B*1502 (16.3%), B*4001 (14.2%), and B*5801 (12.2%). Twenty-nine alleles were detected in NPC patients with the same five common alleles observed at high frequencies (11.1-46.3%). In addition, the allele frequencies of B*38021 (11.1%) and B*51012 (12.9%) were high in the patient group compared to controls.

The summarized of HLA-A and HLA-B alleles commonly present in Thai NPC patients which are the main target population was shown in Table 4.

Association between certain HLA class I alleles with NPC

When the frequency of HLA-A and B antigens in NPC patients and normal individuals was compared, significant associations between NPC and 2 HLA antigens were observed, as summarized in Table 5. Specifically, the frequencies of A2 and B46, were significantly increased in NPC patients (58 and 30% vs. 38 and 14%, $p = 0.02$ and $p = 0.03$, respectively).

When the frequency of high-resolution HLA-B alleles in NPC patients and normal individuals was compared, significant associations between NPC and three HLA-B alleles were observed, as summarized in Table 6. In conclusion, this study reported a protective B*44032 allele and two susceptible, B*4601 and B*51012 alleles, for NPC in Thai population.

Sequence analysis of HLA class I-restricted LMP1 CTL epitopes in EBV isolates from Thai NPC

In the previous studies, target epitopes in LMP1 were identified using CTLs reactivated with the reference type1 EBV strain, B95.8. However, if such epitopes are to form the basis of an effective CTL therapy for EBV-associated malignancies, we must first determine the extent to which these epitope sequences are conserved among other EBV strains from different world populations. Therefore, a panel of Thai NPC biopsies were sequenced across the DNA regions encoding for the LMP1 epitopes and compared with previous data of EBV isolates from other reports. Specifically, CAO strain is the EBV isolate derived from NPC biopsy from Chinese patient (Figure 6). C15 strain is the EBV isolate derived from a white Mediterranean NPC patient. Raji strain is the EBV isolate derived from an African Burkitt's lymphoma cell line.

A detailed summary of the sequence analysis of four previously identified LMP1 epitopes (ALLVLYSFA, YLLEMLWRL, LLLIALWNL, SDSNSNEGR and NEGRHHLLV) is presented in Table 7. The HLA A2-restricted (ALLVLYSFA and LLLIALWNL) and HLA

B31/33 & B61-restricted (SDSNSNEGR and NEGRHHLLV) epitopes were highly conserved in virus isolates from various sources (Table 7). Only a small number of virus isolates showed minor variation within the epitope sequences. However, data from Thai NPC isolates revealed unique sequence which resulted in amino acid substitution. For example, the substitution of serine instead of isoleucine at position 4 in the LLL[S-> I] ALWNL epitope. The substitution of threonine instead of serine at position 1 in the [T-> S]DSNSNEGR epitope. And the substitution of arginine instead of histidine at position 6 in the NEGRH[R->H]LLV.

Sequence analysis of the HLA A2-restricted epitope, YLLEMLWRL in EBV
In Thai isolates, leucine at position 2 and methionine at position 5 were substituted for phenylalanine and isoleucine, respectively (YFLEILWRL). No wild-type sequence was detected in any of the isolates from South-East Asia. A unique nucleotide substitution of A instead of G at position 12 was also observed although resulted in a silent mutation.

The screening for T cell responses using a set of overlapping peptide from LMP1

CTL epitopes were identified by their ability to stimulate T cell response in the ELISPOT assay. First, each 20-mer peptide was tested with lymphocytes in a single well. Twenty-one healthy donors who carry at least one common HLA allele described in NPC patient as shown in table 4 were recruited for this study. List of donor and their HLA types was shown in table 8.

Out of 21 donors, only 2 donors gave positive result to one overlapping peptide designated peptide#28 (SNSNEGRHHLLVSGAGDD). Specifically gamma-interferon ELISPOT reaction from donor AT (HLA-A11, A31; B13, B51) gave 60 spot forming cells (SFC) / PBMC 10^6 cells and reaction from donor SP (HLA-A11, A24; B18, B27) gave 20 SFC/million cells (Table 9). None of the other overlapping peptides can significantly stimulate gamma-interferon production from all the 21 donors. These positive result with peptide#28 was confirmed by repeating the ELISPOT assay which gave similar results (data not shown).

Peptide#28 contains two previously characterized CTL epitopes, B31/33 and B61-restricted SDSNSNEGR and NEGRHHLLV, respectively. We further tested PBMC from the 2 donors with these 2 well-defined epitopes. However, no spot were detected from either donor against SDSNSNEGR or NEGRHHLLV (table 9) suggesting that other minimal epitopes might be important.

Due to the limited budget, we apply bioinformatic technology to further predict a minimal epitope from peptide#28 instead of the original approach of making 9-mer peptides spanning the entire length of the 18-mer peptide. Using program "Binding Motif Scanner" from the HLA molecular Immunology database (http://hiv.basic.nwu.edu/HLA/Reports/DoMotifList.cfm), we search the entire amino acid sequence of LMP1 for putative CTL epitopes restricted to HLA alleles of the 2 donors (A11, A24, A31, B27, B51). However there are no binding motif of HLA-B13, B18 available in the database. Summary of the predicted result was shown in table 10. A number of putative epitopes (35 distinct sequences) were predicted. However, only 2 sequences were lying within peptide#28, indicated as bold letter in table 10 as B27-restricted GRHHLLVSG and B51-restricted EGRHHLLV.

Table 1. HLA-A frequencies in patients with NPC and healthy controls from Thailand

HLA antigen	NPC Patients (N =53)		Controls (N = 70)	
	n	AgF %	n	AgF %
A1	3	5.6	3	4.2
A2	31	58.5	27	38.5
A3	2	3.7	3	4.2
A9 (A23)	0	0	0	0
A9 (A24)	14	26.4	30	42.8
A10 (A25)	0	0	0	0
A10 (A26)	4	7.5	1	1.4
A10 (34)	0	0	0	0
A11	23	43.3	34	48.6
A19 (A29)	0	0	1	1.4
A19 (A30)	1	1.8	4	5.7
A19 (A31)	0	0	2	2.8
A19 (A32)	0	0	0	0
A19 (A33)	17	32	24	34.3
A19 (A74)	2	3.7	0	0
A28	1	1.8	0	0
A36	0	0	0	0

N = the total number of individuals studied in either patient or control group

n = the number of individuals positive for each antigen

Table 2. HLA-B frequencies in patients with NPC and healthy controls from Thailand

HLA antigen	NPC Patients (N =53)		Controls (N = 70)	
	n	AgF %	n	AgF %
B5 (B51)	0	0	0	0
B5 (B52)	4	7.5	5	7.1
B7	2	3.8	5	7.1
B8	0	0	1	1.4
B12 (B44)	5	9.4	14	20
B12 (B45)	0	0	0	0
B13	10	18.8	16	22.8
B14	0	0	1	1.4
B15 (B62)	5	9.4	7	10
B15 (B75)	6	11.3	5	7.1
B15 (B76)	0	0	0	0
B15 (B77)	0	0	2	2.8
B16 (B38)	4	7.5	4	5.7
B16 (B39)	1	1.8	0	0
B17*	1	1.8	0	0
B17 (B57)	2	3.8	7	10
B17 (B58)	13	24.5	8	11.4
B18	6	11.3	4	5.7
B22 (B54)	1	1.8	2	2.8
B22 (B55)	1	1.8	1	1.4
B22 (B56)	1	1.8	3	4.3
B27	1	1.8	5	7.1
B35	4	7.5	9	12.8
B37	1	1.8	0	0
B40 (B60)	10	18.8	16	22.8
B40 (B61)	4	7.5	3	4.3
B42	0	0	1	1.4
B46	16	30.2	10	14.2

B47	0	0	0	0
B48	0	0	1	1.4
B70	1	1.8	0	0

N = the total number of individuals studied in either patient or control group

n = the number of individuals positive for each antigen

* Subtype of B17 could not be interpreted in one individual, who has both B15 and B17, because the key sera that differentiate B17 subtype is duo-specific for B15 and B17 subtype (B57).

Table 3. High resolution HLA-B Allele frequencies in patients with NPC and healthy controls from Thailand

HLA-B	NPC Patients		Controls	
Ailele	Count (2N =108)	%	Count (2N = 98)	%
B*0705/6	1	1.8	2	4.0
B*1301	4	7.4	4	8.1
B*1302	1	1.8	1	2.0
B*1402	0	0.0	1	2.0
B*1501	3	5.5	1	2.0
B*1502	6	11.1	8	16.3
B*1504	1	1.8	0	0.0
B*1511	1	1.8	1	2.0
B*1517	1	1.8	0	0.0
B*1518	0	0.0	1	2.0
B*1521	0	0.0	1	2.0
B*1525	1	1.8	2	4.0
B*1532	0	0.0	1	2.0
B*1801	1	1.8	2	4.0
B*1802	4	7.4	1	2.0
B*2704	0	0.0	2	4.0
B*2706	0	0.0	3	6.1
B*3501	0	0.0	1	2.0
B*3503	1	1.8	1	2.0
B*3505	1	1.8	1	2.0
B*3701	1	1.8	1	2.0
B*38021	6	11.1	2	4.0
B*3804	0	0.0	1	2.0
B*3901	1	1.8	0	0.0
B*3909	1	1.8	3	6.1
B*4001	13	24.1	7	14.2
B*4002	3	5.5	2	4.0
B*4006	0	0.0	2	4.0
B*44032	6	11.1	14	28.5
B*4601	25	46.3	8	16.3

B*4801	1	1.8	0	0.0
B*51011	5	9.2	3	6.1
B*51012	7	12.9	0	0.0
B*52011	3	5.5	5	10.0
B*5502	1	1.8	2	4.0
B*5604	1	1.8	0	0.0
B*5801	7	12.9	6	12.2
B*5401	1	1.8	4	8.1
B*5701	0	0.0	3	6.1
B*7021	0	0.0	1	2.0

N = the total number of individuals studied in either patient or control group

Table 4. HLA class I alleles commonly present in Thai NPC population

HLA allele	Allele frequency in Thai NPC patients
HLA-A2 [a]	58%
HLA-A11	43%
HLA-A24	26%
HLA-A33	32%
HLA-B75	11%
HLA-B44	9-11%
HLA-B46	30-46%
HLA-B58	12-24%
HLA-B60	19-24%

[a] Common HLA-A2 subtypes in Thai population are A*0203 and A*0207 (Vejbaesya et al., 1998)

Table 5. HLA-A and B antigens that demonstrated significant associations with NPC

HLA	NPC (N = 53)		Controls (N = 70)		+/- association	p value
	n	%	n	%		
HLA-A2[a]	31	58	27	38	+	0.02
HLA-B46[b]	16	30	10	14	+	0.03

N = the total number of individuals studied in either patient or control group

n = the number of individuals positive for each antigen

[a] $\chi^2 = 6.4$, p = 0.02, OR = 2.24, 95% CI = 1.02-4.97

[b] $\chi^2 = 7.9$, p = 0.03, OR = 2.59, 95% CI = 0.98-6.95

Table 6. HLA-B alleles that demonstrated significant associations with NPC

HLA-B*	NPC (N = 54)		Controls (N = 49)		+/- association	p value
	n	%	n	%		
44032[a]	5	9	14	29	-	0.01
4601[b]	21	39	7	14	+	0.005
51012[c]	6	11	0	0	+	0.02

N = the total number of individuals studied in either patient or control group

n = the number of individuals positive for each allele

[a] χ^2 = 6.4, p = 0.01, OR = 0.26, 95% CI = 0.07-0.85

[b] χ^2 = 7.9, p = 0.005, OR = 3.8, 95% CI = 1.34-11.82

[c] Fisher's exact, p = 0.02

Figure 2. Example of complete sequences of nucleotide bases and amino acids from latent membrane protein-1 (LMP1) gene, CAO strain which is an EBV isolated from NPC biopsy from Chinese patients.

>gi|11136612|gb|AF304432.1|AF304432 Human herpesvirus 4 latent membrane protein-1 (LMP1) gene, complete cds

ATGGAACGCGACCTTGAGAGGGGCCCACCGGGCCCGCCACGGCCCCCTCTAGGAC
CCCCCCTCTCCTCTTCCATAGGCCTTGCTCTCCTTCTCCTGCTCTTGGCGCTACTGT
TCTGGCTGTATATCGTTATGAGTGACTGGACTGGAGGAGCGCTCCTTGTCCTCTATT
CCTTTGCTCTCATGCTTATTATTATCATTCTCATCATCTTTATCTTCAGAAGAGACCTT
CTCTGTCCACTTGGAGGCCTTGGTCTACTCCTACTGATGAGTAAGTATTACACCCTTT
GCCCCCCACCCCCTTTCCCTTACGCTTCCTTCTCTAACGCACTTTCTCCTCTTTCCCC
AGTCACCCTCCTACTCATCGCTCTCTGGAATTTGCACGGACAGGCATTGTACCTTGG
AATTGTGCTGTTCATCTTTGGCTGCTTACTTGGTAAGATCTAACATTCCCTAGGACTT
ATTTACCACACCCTCACCTTTCCAGCCCTAACACTCTTTTTTTCAACGCAGTCTTAGG
TCTCTGGATCTACTTCTTGGAGATTCTCTGGCGGCTTGGTGCCACCCTCTGGCAGCT
TTTGGCCTTCATCCTAGCCTTCTTCCTAGCCATCATCCTGCTTATTATTGCTCTCTATC
TACAACAAAACTGGTGGACTCTATTGGTTGATCTCCTTTGGCTCCTCCTGTTTATGGC
CATTTTAATCTGGATGTATTATCATGGACCACGACACACTGATGAACACCACCACGAT
GACTCCCTCCCGCACCCTCAACAAGCTACCGACGATTCTAGCCATGAATCTGACTCT
AACTCCAACGAGGGCAGACACCACCTGCTCGTGAGTGGGGCCGGCGACGGACCCC
CACTCTGCTCTCAAAACCTAGGCGCACCTGGAGGTGGTCCTGACAATGGCCCACAG
GACCCTGACAACACTGATGACAATGGCCCACAGGACCCTGACAACACTGATGACAAT
GGCCCACAGGACCCTGACAACACTGATGACAATGGCCCACAGGACCCTGACAACAC
TGATGACAATGGCCCACAGGACCCTGACAACACTGATGACAATGGCCCACAGGACC
CTGACAACACTGATGACAATGGCCCACAGGACCCTGACAACACTGATGACAATGGC
CCACATGACCCGCTGCCTCATAACCCTAGCGACTCTGCTGGAAATGATGGAGGCCC
TCCAAATTTGACGGAAGAGGTTGCAAACAAAGGAGGTGACCGGGGCCCGCCTTCGA
TGACAGACGGTGGCGGCGGTGATCCACACCTTCCTACGCTGCTTTTGGGTACTTCT
GGTTCCGGTGGAGATGATGACGACCCCCACGGCCCAGTTCAGCTAAGCTACTATGA
CTAA

>gi|11136613|gb|AAG31283.1|AF304432_1 latent membrane protein-1 [Human herpesvirus 4]

MERDLERGPPGPPRPPLGPPLSSSIGLALLLLLLALLFWLYIVMSDWTGG**ALLVLYSFA**L

ML**I**I**I**L**I**IFIFRRDLLCPLGGLGLLLLM**IT****LLLIALWNL**HGQALYLGIVLFIFGCLLVLGLW**I****YFL**

EI**LWRL**GATLWQLLAFILAFFLA**I**ILL**I**IALYLQQNWWTLLVDLLWLLLFMA**I**LIWMYYHGPR

HTDEHHHDDSLPHPQQATDDSSHE**SDSNSNEGRHHLLV**SGAGDGPPLCSQNLGAPGG

GPDNGPQDPDNTDDNGPQDPDNTDDNGPQDPDNTDDNGPQDPDNTDDNGPQDPDN

TDDNGPQDPDNTDDNGPQDPDNTDDNGPHDPLPHNPSDSAGNDGGPPNLTEEVANK

GGDRGPPSMTDGGGGDPHLPTLLLGTSGSGGDDDDPHGPVQLSYYD

Note: Bold letters indicate the position of five CTL epitopes described in the sequencing result in this study.

Table 7. Sequences of HLA class I-restricted LMP1 epitopes in EBV isolates from Thai NPC biopsy compared to various EBV strains characterized previously

Virus origin	Epitope sequence	HLA restriction	Number of isolates
B95.8[a]	GCC CTC CTT GTC CTC TAT TCC TTT GCT A L L V L Y S F A	HLA-A2	
CAO[b]	--G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---		
C15[c]	--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---		
Raji[d]	--- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- A --- ---		
Thai	--G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---		3
Thai	--G --- --- --- --- --- G-- --- --- --- --- --- --- --- --- A --- ---		2
B95.8[a]	CTC CTA CTC ATC GCT CTC TGG AAT TTG L L L I A L W N L	HLA-A2	
CAO[b]	--G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---		
C15[c]	--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---		
Raji[d]	--- --G --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---		
Thai	--- --G --- -G- --- --- --- --- -- --- --- --- --- --- --- --- --- ---		1

	- _ _ _ S _ _ _ _ _		
Thai	--- --- --- --- --- --- --- --- --- _ _ _ _ _ _ _ _ _		1
B95.8[a]	**TAC TTA TTG GAG ATG CTC TGG CGA CTT** **Y L L E M L W R L**	HLA-A2	
CAO[b]	--- --**C** --- --- --**T** --- --- --**G** --- _ **F** _ _ **I** _ _ _ _		
C15[c]	--- --- --- --**C** --**T** --- --- --- --- _ _ _ **D** **I** _ _ _ _		
Raji[d]	--- --- --- --- --**T** --- --- --- --- _ _ _ _ **I** _ _ _ _		
Thai	--- --**C** --- --- --**T** --- --- --**G** --- _ **F** _ _ **I** _ _ _ _		1
Virus origin	Epitope sequence	HLA restriction	Number of isolates
Thai	--- --**C** --- --**A** --**T** --- --- --**G** --- _ **F** _ _ **I** _ _ _ _		1
B95.8[a]	**TCT GAC TCT AAC TCC AAC GAG GGC AGA** **S D S N S N E G R**	HLA-A31/A33	
CAO[b]	--- --- --- --- --- --- --- --- --- _ _ _ _ _ _ _ _ _		
C15[c]	--- --- --- --- --- --- --- --- --- _ _ _ _ _ _ _ _ _		

Raji[d]	--- --- --- --- --- --- --- --- --- – – – – – – – – –		
Thai	--- --- --- --- --- --- --- --- --- – – – – – – – – –		10
Thai	A-- --- --- --- --- --- --- --- --- T – – – – – – – –		1
B95.8[a]	AAC GAG GGC AGA CAC CAC CTG CTC GTG N E G R H H L L V	HLA-B61	
CAO[b]	--- --- --- --- --- --- --- --- --- – – – – – – – – –		
C15[c]	--- --- --- --- --- --- --- --- --- – – – – – – – – –		
Raji[d]	--- --- --- --- --- -t- --- --- --- – – – – – L – – –		
Thai	--- --- --- --- --- --- --- --- --- – – – – – – – – –		10
Thai	--- --- --- --- --- -G- --- --- --- – – – – – R – – –		1

[a] B95.8 is a reference type I EBV strain isolated from a marmoset monkey lymphocyte.

[b] CAO is a strain derived from a Chinese NPC patient.

[c] C15 is a strain derived from a white Mediterranean NPC patient.

[d] Raji is a strain derived from an African Burkitt's lymphoma cell line.