Table 8. List of donors with HLA types that were included in the overlapping peptide screening test

ID	HLA-A	HLA-B
PR	A2, A24	B35, B60
NA	A11, A24	B27, B60
WI	A24, A33	B13, B44
CH	A24	B56, B75
AT	A11, A31	B13, B51
PA	A2, A11	B60
WE	A2, A33	B44, B46
SP	A24, A11	B18, B27
SU	A2, A3	B13, B61
SO	A1, A24	B57, B35
AN	A3, A33	B75, B57
SM	A2	B57
PO	A24	B27, B60
SA	A11	B27
CA	A11, A33	B44, B61
OR	A2, A33	B51, B58
SB	A11, A33	B44, B52
IN	A11, A24	B35, B38
KA	A11, A26	B8, B60
UB	A11, A26	B52, B58
PN	A2, A24	B27, B52

Table 9. Summarized result of the overlapping peptide screening from LMP1 sequences

ID	HLA-A	HLA-B	peptide	ELISPOT result (SFC/ million)
AT	A11, A31	B13, B51	Peptide#28: SNSNEGRHHLLVSGAGDD	60
			SDSNSNEGR	0
			NEGRHHLLV	0
SP	A24, A11	B18, B27	Peptide#28: SNSNEGRHHLLVSGAGDD	20
			SDSNSNEGR	0
			NEGRHHLLV	0

SFC = spot forming cell

Table 10. List of potential epitopes predicted by “Binding Motif Scanner” in HLA molecular Immunology database

HLA (Serotype)	HLA (Genotype)	Motif	Potential epitopes
A11	A*1101	XXXXXXXX[K]	NLTEEVANK
A24	-	X[Y]XXXXXX[I,L,F]	LYSFALMLI
			LYLGIVLFI
A31	A*3101	XXXXXXXX[R]	ERGPPGPPR
			IILIIFIFR
			ILIIFIFRR
			IYFLEILWR
			IWMYYHGPR
			SDSNSNEGR
			EVANKGGDR
B27	-	X[R]XXXXXXX	ERDLERGPP
			ERGPPGPPR
			PRPPLGPPL
			FRRDLLCPL
			RRDLLCPLG
			WRLGATLWQ
			PRHTDEHHH
			GRHHLLVSG
			DRGPPSMTD
B51	B*5101	X[A,P,G]XXXXXX[F,I]	LGPPLSSSI
			LALLFWLYI
			GALLVLYSF
			FALMLIIII
			HGQALYLGI
	B*5102	X[A,P,G]XXXXXX[V,I]	LGPPLSSSI
			LALLFWLYI
			FALMLIIII
			HGQALYLGI

		GPPNLTEEV
B*5103	**X[A,P,G]XXXXX[V,F,I]**	**GPPLSSSI**
		FALMLIII
		LGLLLLMI
		QALYLGIV
		LGIVLFIF
		LAFILAFF
		LAFFLAII
		LAIILLII
		EGRHHLLV
		PPNLTEEV

Note: Bold letters indicate the potential epitopes since they are located within peptide#28.

DISCUSSION

HLA distribution data from Thai NPC patients is useful since vaccine development including T cell epitopes restricted to these common alleles will benefit the majority of affected population. As demonstrated in this study, HLA typing data was helpful in the process of selecting donors as listed in table 8. Interestingly, the frequencies of certain HLA alleles were significantly different between NPC patient and normal control group. In conclusion, this study reported 2 susceptible, A2 and B46 antigens, for NPC in Thai population. In the allelic level a protective B*44032 allele and two susceptible, B*4601 and B*51012 alleles, were also identified for Thai NPC population.

A lower incidence of B*44032 in the patient group was noticed (9 vs. 29%, $p < 0.01$) similar to a previous report in East Africa in which B44, characterized by serological typing, was reported as a resistance antigen in NPC (Moore et al., 1983). Interestingly, some CTL epitopes from EBV antigens have been identified as restricted by B44 (Khanna et al., 1997). Studies of those epitopes that contributing to the protective effect of the protective allele might be useful in vaccine development. The frequencies of another two alleles, B*4601 and B*51012, were significantly increased in NPC patients (39 and 11% vs. 14 and 0%, $p<0.005$ and $p<0.02$, respectively). As mentioned above, the association with B46 has been consistently observed in Chinese populations (reviewed in Ren and Chan, 1996; Simons et al., 1974 and 1975; Chan et al., 1983 and 1985). One previous report of 20 Thai NPC patients also demonstrated a positive association of NPC with B46 ($p<0.05$) (Chan et al., 1985). Our study has confirmed that result suggesting that genetic susceptibility of NPC to in the Thai population is likely similar to the Chinese population. The restricted-antigen binding properties of the B*4601 molecule (Barber et al., 1996) might be one explanation for its link to susceptibility to NPC. Interestingly, no EBV epitopes restricted by B46 have been reported so far and further studies are required to prove this hypothesis. Although there are some relationships between HLA types and NPC, the exact nature of this association is not yet clear. It is more likely that HLA antigens are not involved in the causation of the disease but are very closely linked to the "disease susceptibility genes". In fact, many studies supported this latter theory (Lu et al., 1990; Ooi et al., 1997). However, no major susceptibility genes of NPC have been identified so far.

Previous studies have suggested that LMP1 sequences in different geographic regions of the world display a very high degree of variation. This genetic variation has been considered as a major impediment towards the use of LMP1 as a potential immunotherapeutic target for the treatment of relapsed NPC. Hence it was important to determine the extent to which the B95.8 derived LMP1 epitope sequences are conserved in EBV isolates from different geographic regions of the world. Studying a panel of EBV isolates representative from African (Raji) and Chinese NPC (CAO, C15) as well as Thai NPC biopsies, we found that T cell epitope sequences from LMP1 were generally well conserved. Four of the five epitopes sequenced here showed only minor sequence variation. However, some unique nucleotide substitution which resulted in amino acid substitution were identified from Thai NPC isolates within these relatively conserved epitopes. Although the effect of these changes against CTL activity remains to be further investigated. These findings stress the necessitation of specific epitope characterization from each endemic area.

The only epitope with major sequence divergence was the HLA A2 supertype-restricted YLLEMLWRL epitope, which displayed an interesting pattern of sequence diversity in EBV isolates from different geographic regions. The analysis of the genetic variants of the YLLEMLWRL epitope indicated that the variant sequence (YFLEILWRL) is prevalent in South-East Asia. Although the precise reason/mechanism for such a high degree of genetic variation within this epitope is not known, it is possible that mutation within this epitope in the isolates from South-East Asia, where NPC is endemic, may provide an advantage in protecting these isolates from the EBV-specific CTL response.

A low frequency of positive responses against LMP1 have been reported in several studies (Khanna et al., 1998; Leen et al., 2001; Meij et al., 2002; Whitney et al., 2002). Recent study by Meij et al., 2002 using the same strategy of overlapping peptide screening in healthy Caucasian donors detected positive response only in 9 out of 50 donors (18%). In addition, the number of cells reactive to LMP1 were very low similar to our study. In summary, this protein is poorly immunogeneic. The explanation for this weakly immunogeneic is not understood. It is possible that LMP1 may has immunosuppressive properties that favor the induction of T cell anergy (Dukers et al., 2000). However, to target EBV-positive NPC tumor, such small response against LMP1 should be amplified, either by active immunization or by *in vitro* expansion and re-infusion. Therefore, more donors should be included to obtain more putative CTL epitopes of LMP1.

SUGGESTION FOR FURTHER WORK

Further identification of novel CTL epitopes from LMP1 using a larger panel of donor should be pursued with the use of bioinformatic technology e.g., binding motif scanner. The characterization CTL epitopes in Thai population from other candidate protein such as LMP2 is also needed by applying the same strategy as this study. In addition, increasing interest has been focusing on T helper epitopes as well since the addition of T helper epitope with CTL epitopes should increase the efficiency of the immunotherapy. Therefore, the characterization of T helper epitopes restricted to common HLA class II alleles is another subject of interest for future work.

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OUTPUT จากโครงการวิจัยที่ได้รับทุนจากสกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

- Pimtanothai N, Charoenwongse P, Mutirangura A, Hurley CK. "Distribution of HLA-B alleles in nasopharyngeal carcinoma patients and normal controls in Thailand." Tissue Antigens 2002; 59: 223-225

นอกเหนือจากผลงานตีพิมพ์ที่ได้จากโครงการนี้แล้ว ตัวอย่าง DNA จากผู้ป่วยมะเร็งโพรงหลังจมูกจากโครงการนี้ยังถูกนำไปใช้ศึกษาในโครงการวิจัยอื่น และมีผลงาน คือ

- Hirankarn (Pimtanothai) N., Kimkong I, Mutirangura A. "HLA-E and genetic susceptibility to nasopharyngeal carcinoma" Manuscript in Preparation (Plan to submit to Tissue Antigen)

2. การเสนอผลงานในที่ประชุมวิชาการ

- Pimtanothai NH, Kimkong I, Mutirangura A 2002 HLA-E polymorphism in Thai patients with nasopharyngeal carcinoma FIMSA Advanced course and conference on "Molecular Mechanisms of Infection and Immunity" Ayuthaya, Thailand. October 23, 2002.

APPENDIX

Brief communication

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Distribution of HLA-B alleles in nasopharyngeal carcinoma patients and normal controls in Thailand

Key words:
HLA-B, nasopharyngeal carcinoma

Acknowledgments:
The authors are deeply indebted to the staff of the Department of Otolaryngology and the Radiotherapy section, Department of Radiology, Chulalongkorn University, for the recruitment of patients and collection of materials. We also thank Mrs Ratchada Intwatana for DNA preparation. This work was supported by the Molecular Biology Research Fund, Faculty of Medicine, Chulalongkorn University, the Thailand Research Fund, and US office of Naval research N00014-94-0049.

Received 4 October 2001, revised 14 February 2002, accepted for publication 18 February 2002

Tissue Antigens 0001 2815

Tissue Antigens 2002 59 223–225
Printed in Denmark.

Abstract: HLA-B frequencies in 54 unrelated nasopharyngeal carcinoma (NPC) patients and 49 healthy random controls in Thailand were investigated by direct DNA sequencing. Similar to previous reports in Chinese NPC patients, HLA-B*4601 was observed at a greater frequency in patients (21/54 (40%)) compared to controls (7/49 (14%)). An increase in HLA-B*51012 was also demonstrated. B*51012 was present in 6/54 (11%) NPC patients but was not observed (0%) in controls. B*44032 was associated with a decreased risk. Five out of 54 (9%) NPC patients had B*44032 compared to 14/49 (29%) in the control group.

Nasopharyngeal carcinoma (NPC) is a tumor affecting the epithelial lining in the head and neck region. It is one of the most common cancers in Asia, with the highest incidence rate in South China and intermediate incidence rate in South-east Asia (1). This tumor is quite rare in the Western population. Multiple factors have been reported to be involved in the pathogenesis of this disease including EBV infection, environmental carcinogens (e.g., cigarette smoke, certain foods), and genetic factors (2). HLA is one of the genetic factors reported as having a significant association with NPC (1). Certain HLA antigens or haplotypes, mostly identified using serological techniques, have been associated with either increased or decreased risk in various studies. For example, HLA-A2/B46 haplotypes were consistently reported to be positively associated with NPC in Chinese populations living in different countries (e.g., Singapore, China, Hong Kong, Malaysia, California, U.S.A) (1, 3–6). HLA-B58 is another NPC-associated antigen observed at a higher frequency in Chinese and Malay patients (7) while HLA-A11 was observed at a lower frequency among Chinese NPC patients (6, 7). Studies of HLA in other low incident populations gave more variable results (5–12).

The underlying mechanisms of these associations have not been elucidated. One explanation is the existence of an NPC susceptible gene closely linked to the HLA region which was suggested from an

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HLA-B allele frequencies in patients with NPC and healthy controls from Thailand

	NPC Patients		Controls	
HLA-B Allele	Count (2 N = 108)	%	Count (2 N = 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 the patient or control group

Table 1

HLA-B alleles that demonstrated significant associations with NPC

	NPC (N = 54)		Controls (N = 49)			
HLA-B*	n	%	n	%	± association	*P*-value
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] $\chi^2 = 6.4$, $P = 0.01$, OR = 0.26, 95%CI = 0.07–0.85

[b] $\chi^2 = 7.9$, $P = 0.005$, OR = 3.8, 95%CI = 1.34–11.82

[c] Fisher's exact $P = 0.02$

Table 2

HLA study of 30 sibling pairs (13). A more recent study, using microsatellite markers, predicted that this putative NPC susceptible gene, not yet identified, is close to the D6S1624 marker (14). However, the ability of certain HLA molecules to present EBV antigens might also contribute to the observed HLA associations. Moreover, with the increasing interest in epitope-based immunotherapy in EBV-related tumors including NPC, the distribution of HLA alleles in NPC populations will provide useful information for epitope selection i.e., by including the epitopes restricted to common HLA allelic products in the target population. In this study, we investigated the distribution of HLA-B alleles in Thai NPC patients compared to normal controls to identify the HLA-B alleles associated with NPC in Thailand.

The study population included 54 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 years). All of these patients were positive for the EBNA1 gene in tumor cells as detected by PCR, as reported previously (15, 16). Forty-nine healthy unrelated Thai individuals served as ethnically and geographically matched controls. All patients and controls were typed for the HLA-B locus 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 (17, 18). The allele frequencies were determined by direct counting based on the assumption that individuals carrying a single allele were homozygous. The significance of differences between the two groups was analyzed by the chi-square test. Fisher's exact tests were applied if the expected frequency was less than 5.

The distribution of HLA-B alleles between the two groups is shown in Table 1. A total of 34 HLA-B alleles was observed in the Thai control group, including one new allele (B*3804) (19). 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. This information is useful as vaccine development including T cell epitopes restricted to these common alleles will benefit the majority of affected population. When the frequency of 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 2. Specifically, a lower incidence of B*44032 was noticed in the patient group (9 vs 29%, $P<0.01$) which was similar to a previous report in East Africa, in which B44, characterized by serological typing, was reported as a resistance antigen in NPC (10). Interestingly, some CTL epitopes from EBV antigens have been identified as restricted by B44 (20). Studies of these epitopes that contribute to the protective effect of the protective allele might be useful in vaccine development. The frequencies of another two alleles, B*4601 and B*51012, were significantly increased in NPC patients (39 and 11% vs 14 and 0%, $P<0.005$ and $P<0.02$, respectively). As mentioned above, the association with B46 has been consistently observed in Chinese populations (1, 3–6). One previous report of 20 Thai NPC patients also demonstrated a positive association of NPC with B46 ($P<0.05$) (6). Our study has confirmed that genetic susceptibility of NPC to in the Thai population is likely to be similar to the Chinese population. The restricted-antigen binding properties of the B*4601 molecule (21) might be one explanation for its link to susceptibility to NPC. Interestingly, no EBV epitopes restricted by B46 have been reported so far and further studies are required to prove this hypothesis. No association with B58 was demonstrated in this study; however, in a previous study, B58 association was only seen in newly diagnosed patients and its frequency is very low in long-term survivors (1), which were not identified in this study.

In conclusion, this study reported a protective B*44032 allele and two susceptible alleles, B*4601 and B*51012, for NPC in Thai population.

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HLA-E and genetic susceptibility to nasopharyngeal carcinoma

Short Title: HLA-E and nasopharyngeal carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) has been known to be associated with HLA class I region. The aim of this study was to investigate the association between HLA-E and genetic susceptibility to nasopharyngeal carcinogenesis by comparing the frequencies of HLA-E alleles in 104 Thai patients with NPC and 100 healthy controls. HLA-E typing was performed using PCR-SSOP method. DNA sequencing was used to confirm the accuracy of PCR-SSOPs results. The frequency of the HLA-E*0103, 0103 genotype, but not others, was increased in the patients compared to controls ($p = 0.0141$, OR = 2.11, 95% CI = 1.15-3.88). A significant increase in the frequency of the HLA-E*0103 allele was found in the patients compared to normal controls ($p = 0.0076$, OR = 1.78, 95% CI = 1.16-2.74). These might suggest a possible important role for HLA-E in NPC development, possibly via NK cell or CTL function.

Key words: Nasopharyngeal carcinoma; HLA class I; HLA-E; PCR-SSOP; NK cell: CTL cell

Introduction

Nasophayngeal carcinoma (NPC) is a geographically restricted tumor of epithelial cell lining nasopharynx (1,2). The tumor is rare among European and North American Caucasians (less than 1 per 100,000). However, it is more common in many Asian countries, especially common in Southern China (30 - 50 per 100,000) (3). 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 (4-6). 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 factor (7-9). HLA associations have been reported in NPC. Several studies have been reported that HLA-A2, HLA-B46, HLA-B58 and HLA-B*51012 were associated with NPC (1,2,7). However, The explanations of these associations are still unclear. Another possibility is that there may be another disease susceptibility gene that 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 (11). 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. In that study, they found that 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 (12). In our study, we are interested in the HLA-E gene that is in close proximity to D6S1624 microsatellite locus.

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 (13). Transcripts of this gene have been found in a variety of different tissues (14). HLA-E may be involved in the regulation of Natural killer (NK) cell function by presenting leader peptide derived from HLA-A, B, C and G molecules to interact with NK cell receptors and affects inhibiting or activating function of NK cell-mediated lysis (15-18). Recent study has shown that different HLA-E alleles can affect different functions of natural killer cells (19). In addition, several observations raise the possibility that HLA-E might plays an important role in the regulation of cytotoxic T lymphocyte (CTL) function, as CD94/NKG2 receptors are expression on a subset of CTL cell function (20,21). 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 (22). The aim of this study was to investigate the association between HLA-E and genetic susceptibility to nasopharyngeal carcinogenesis by comparing the frequencies of HLA-E alleles in 104 Thai patients with NPC and 100 healthy controls.

Material and methods

Subjects

After having obtained the subjects' informed consent as to purpose of the study blood samples were collected by venipuncture. This study has been approved by The Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. One hundred and four Thai patients with NPC who attended at King Chulalongkorn Memorial Hospital from 1994 to 2001 and 100 control samples from healthy blood donors in Thai Red Cross Society. The tumor of every patient was histologically ascertained as NPC type II or III, according to WHO classification. These patients' ages ranged from 5 to 82 years (mean 41.2) at the time of diagnosis whereas the control subjects ages ranged from 18 to 59 years (mean 34.7). Males outnumber females among the cases was 1.8: 1 and among the controls 1.4: 1, respectively.

Extraction and amplification of genomic DNA

Molecular genetic analysis was performed on genomic DNA, obtained from peripheral blood lymphocytes using standard phenol-chloroform extraction procedure as previously described (23). Exon 2 and exon 3 were separately amplified by polymerase chain reaction (PCR) using primers HLA-E.2F [5' GAA ACG GCC TCT ACC GGG AGT AG 3']- HLA-E.2R [5' GTT CCG CAG CCT TGG GGT GAA TC 3'] and HLA-E.3F [5' CGG GAC TGA CTA AGG GGC 3']-HLA-E.3R [5' AGC CCT GTG GAC CCT CTT 3'] (24,25).

HLA-E typing

Twelve SSOPs (Sequence specific oligonucleotide probes) were used for oligotyping of HLA-E alleles. Ten SSOPs were described previously (25). Two SSOPs of codon150 were designed in this study [GATGCCTCTGAGGCG and GATGCTTCTGAGGCG]. The DNA typing of HLA-E gene was performed by dot-blot hybridization method. The SSOPs were labeled with γ–^{32}P-ATP (Amersham, England) and T4 polynucleotide kinase (New English BioLab, USA). PCR products were spotted onto nylon membrane (Hybond-N; Amercham, England), immobilized by denaturing solution [1.5 M NaCl and 0.5 M NaOH] and neutralized with neutralizing solution [1.5 NaCl, 0.5 M Tris-HCl (pH 7.2) and 0.5 M EDTA (pH 8.0)]. Prehybridization of the membrane was performed in a hybridization oven (Stuart Scientific, England) 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. Poshybridization washing was done as follow: 5xSSC at 42°c for 10 minutes and twice for 10 minutes at 50°c in the same solution. Hybridization signals were detected by exposure the blots DNA to a phosphor screen and visualized on Phopholmager using ImageQuaNT software (Molecular Dynamics, USA).

DNA sequencing

Sequencing was used to screen for additional polymorphism at other locations besides the one previously characterized and used to confirm the accuracy of PCR-SSOPs results. 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. Approximately 100 ng each of PCR products were sequenced with both directions of exon 2 and exon 3 primers as previously described (24,25). 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.

Statistical analysis

The number of HLA-E alleles was obtained using gene counting. The association between certain alleles of HLA-E gene and NPC was estimated by the statcalc from Epi info version 6 program (http:// www. Cdc.gov/epiinfo/EI6dnjp.htm) to calculate the odds ratio (OR) and 95% confidence interval (CI), Yates' corrected chi-squares and associated p values. P values of <0.05 were considered to be significant.

Results

HLA-E genotypes or alleles of 104 patients with NPC and 100 healthy individuals were investigated by PCR-SSOP method. Sequence results did not reveal any new polymorphism at the other locations besides the one previously characterized. The determination of each allele depends on the presence of specific nucleotide as earlier described (26). 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 missense 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 HLA-E*0103 lineage correspond to HLA-EG [A pattern of nucleotide substitution of HLA-E gene, which is Guanine (G)] at codon 107. The HLA-E*0103 comprised E*01031, E*01032 and E*01033. 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-ET [A pattern of nucleotide substitution of HLA-E gene, which is Thymine (T)] at codon 150. 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 polymorphism which composed of HLA-E*0101, 01031 and 01032. When analyzing the association between genotypes or alleles of HLA-E and NPC, the HLA-E*0103 lineage (E*01031 and E*01032) were combined together because both of them correspond to HLA-EG at codon 107. The codon 107(A/G) of HLA-E is a mutation altering the amino acid arginine to glycine. There was a significant increase in the frequency of the HLA-E*0103, 0103 genotype in the patients compared to the normal controls ($p = 0.0141$, OR = 2.11, 95% CI = 1.15-3.88). None of the other genotypes showed any significant association (Table 1). A significant increase in the frequency of the HLA-E*0103 allele was found in the patients compared to normal controls ($p = 0.0076$, OR = 1.78, 95% CI = 1.16-2.74). The HLA-E allelic frequencies in the patients and normal controls are shown in Table 2.

Discussion

A previous study has shown that the D6S1624 microsatellite is strongly associated with NPC (12). HLA-E is in close proximity to this microsatellite marker and is candidate gene that related with function of NK and CTL cells, which are important immune cells against tumor. In this study, the results showed that the NPC susceptibility is associated with certain HLA-E genotypes and alleles. HLA-E*0103, 0103 genotype and HLA-E*0103 allele are associated with NPC. These might suggest a possible important role for HLA-E in NPC development.

Since NPC is a complex disease, HLA-E gene might be one of several causes for NPC development. In addition, 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-E. 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. 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 NK cells (27,28). According to HLA-E*0103 allele is defined by a missense mutation at codon 107, which change from arginine to glycine. One experiment supporting function of HLA-E*0103 (glycine) 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 (29,30). So, it is possible that HLA-E*0101 (arginine) presents the leader peptide from HLA-A2 leading to no inhibition in NK cell-mediated lysis. Whereas HLA-E*0103 allele with the leader peptide from HLA-A2 will inhibit NK cell-mediated lysis (19). 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 lower significantly than controls (31). This might be the effect of HLA-E107G allele to inhibit NK cell-mediated lysis. Secondly, according to the crystal structure of HLA-E

(32), 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 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 (33). Yet, no direct proof for functional implication of CTL response in the interaction between TCR and each HLA-E allele bind to EBV peptides. 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 (21,34). The functional role of HLA-E-NK cell receptor recognition on CTLs remains unclear. However, future studies would be interesting to test the functional properties of the HLA-E alleles in NK cell or CTL for NPC patients.

Acknowledgments

The study was supported by Ministry of University Affairs (MUA)-CU Thesis Grant, the Molecular Biology Project, Faculty of Medicine, Chulalongkong University and Thailand Research Fund. The authors are deeply indebted to the staff of the Department of Otolaryngology and the Radiotherapy Section, Department of Radiology, Faculty of Medicine, Chulalongkong University. And National Blood Center for the recruitment of patients and collection of materials.

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Table 1 Frequency of HLA-E genotypes in patients with NPC and normal controls

HLA-E genotypes	% of patients (n)	% of controls (n)	P value	OR (95% CI)
0101	9.62 (10)	18 (18)		
0101; 0102	0 (0)	0 (0)		
0101; 0103	39.42 (41)	49 (49)		
0101; 0104	0 (0)	0 (0)		
0102	0 (0)	0 (0)		
0102; 0103	0 (0)	0 (0)		
0102; 0104	0 (0)	0 (0)		
0103	50.96 (53)	33 (33)	0.0141[1]	2.11 (1.15-3.88)
0103; 0104	0 (0)	0 (0)		
Total (n)	104	100		

The number of individuals is given in parentheses.

[1] Chi-square probability for the comparison of HLA-E genotypes in patients and controls.

Table 2 Frequency of HLA-E alleles in patients with NPC and normal controls

HLA-E alleles	% of patients (n)	% of controls (n)	P value	OR (95% CI)
0101	29.33 (61)	42.5 (85)		
0102	0 (0)	0 (0)		
0103	70.77 (147)	57.5 (115)	0.0076[1]	1.78 (1.16-2.74)
0104	0 (0)	0 (0)		
Total (n)	208	200		

The number of individuals is given in parentheses.

[1] Chi-square probability for the comparison of HLA-E alleles in patients and controls.