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The Association Between Latent Membrane Protein-1 and CD80, CD86, MHC-I and CD8 in Advanced Stage Nasopharyngeal Carcinoma

İleri Evre Nazofarinks Karsinomunda Latent Membran Protein-1 ile CD80, CD86, MHC-I ve CD8 antijenlerinin ilişkisi

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Abstract

Introduction: This study aimed to investigate the escape mechanism of tumor cell to host immune system via CD80, CD86, MHC Class I and CD8 in advanced stage nasopharynx carcinoma.

Materials and Methods: This cross-sectional analytical observational study was conducted in 434 nasopharynx carcinoma (NPC) patients who visited Otorhinolaryngology Outpatient clinic of Dr. Moewardi Hospital from 2011 to 2014. The data were obtained from medical record system and histopathologic examination results. The sample collection was obtained by consecutive sampling. The expressions of CD80, CD86, MHC Class I and CD8 were assessed by immunohistochemistry staining (IHC).

Results: Of the total study subjects, we found 32 sample of Grade 3 NPC, and the statistical analysis of these sample revealed that although there was no statistically significant association between the expression of latent membrane protein 1 (LMP1) and CD8 (p=0.556), and expression of CD80, there was a statistically significant association between LMP1 and CD86 (p=0.034). Similarly, although no statistically significant association was found between expression of CD8 and LMP1 (p=0.053), MHC Class I expression was found statistically significantly associated with LMP expression (p=0.012).

Conclusion: The effect of LMP1 on CD8 mediated by CD86, MHC Class I is statistically significant in which the increase of LMP1 expression is followed by the decrease of CD8 expression. Thus it suggests the concept of escape mechanism.

Keywords: Escape mechanism, nasopharyngeal carcinoma, World Healt Organization Type 3 advanced stage, latent membrane protein-1(LMP1), MHC Class I, CD80, CD86

Öz

Giriş: Bu çalışmada, tümör hücrelerinin immün sistemden kaçış mekanizmalarını araştırmak için, ileri evre nazofarinks kanserinde CD80, CD86, MHC Sınıf I ve CD8 antijenlerinin rolü araştırıldı.

Gereçler ve Yöntemler: Bu kesitsel çalışmada Dr. Moewardi Hastanesi Kulak Burun Boğaz Hastalıkları Polikliniğine 2011 ila 2014 yılları arasında başvuran 434 nazofarinks karsinomlu (NFK) hasta incelendi. Veriler, tıbbi kayıt sistemi ve histopatolojik irdelemelerin sonuçlarından kaydedildi. Ardışık örnekler çalışmaya alındı. CD80, CD86, MHC Sınıf I ve CD8 ifadeleri immünohistokimya boyamaları ile saptandı.

Bulgular: Tüm çalışma örnekleri içinden 32 örnek Tip 3 NFK olarak saptandı ve LMP1 ifadesi ile CD8 (p=0.556) ve CD80 ifadesi arasında istatistiksel açıdan anlamlı bir ilişki bulunmamasına karşılık, CD86 ifadesi ile istatistiksel açıdan anlamlı bir bağıntı saptandı (p=0.034). Benzer olarak, LMP1 ifadesi ile CD8 arasında istatistiksel açıdan anlamlı bir ilişki olmamasına karşılık (p=0.053), MHC Sınıf I ifadesi CD8 ifadesi ile istatistiksel açıdan bağıntılı olarak saptandı (p=0.012).

Sonuç: CD8+ hücrelerdeki LMP1'in etkisi CD86 ile ilişkilidir, MHC Sınıf I istatistiksel olarak anlamlıdır ve LMP1 ifadesi arttıkça CD8 ekspresyonu azalış göstermektedir. Bu da LMP1'in bağışıklık sisteminden kaçış mekanizmasında rol oynayabileceğini göstermektedir.

Anahtar Kelimeler: Kaçış mekanizması, nazofarinks karsinomu, Dünya Sağlık Teşkilatı (DSÖ) Tip 3 ileri evre, latent membrane protein 1(LMP1), MHC Sınıf I, CD80, CD86

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Introduction

Nasopharyngeal Carcinoma (NPC) is an epithelial cancer of lateral nasopharyngeal wall which develops metaplasia from cilliary columnar epithelium. The etiologic factors are various, such as genetic predispositions, Ebstein Barr Virus (EBV) infection, environmental factors and food.[1,2]

In Indonesia, the incidence of nasopharyngeal cancer has increased significantly. It becomes the fourth most frequent cancer after ovarian, breast and skin cancers. Generally, patients are admitted to hospital at advanced stage (Stage 3 and 4) and the 5 year survival rate is low. The most common type is World Health Organization (WHO) Grade 3 (55%) followed by Grade 2 (25%) and Grade 1 (20%).[3–6]

EBV has strong and consistent association with Grade 3 NPC which infects more than 90% of the world population. EBV antigen induces the formations of co-stimulator CD80, CD86 and MHC Class I. The signal released by CD28 leads to T cell proliferation. This interaction is an early process of specific immunity which ended with the elimination of the antigen virus by CD8+ T Cells.[7–9]

Chang et al.[10] stated that the lack of B7 co-stimulator molecule (CD80 and CD86) causes cancer cells to develop. Yang et al. reported that the expressions of CD80 and CD86 with ligand CD28 an mRNA levels were lower than those of normal tissue. Ligand of CD28 in APC is B7 (CD80 and CD86) whose gene is located in chromosome 3q13.3–3q2130 that is expressed by B cells, T cells, macrophages and dendritic cells. Co-stimulator of B7 can also bind to CTLA-4 which has an opposite effect in T cell response.[10,11] Costimulation of B7 prevents the T cell energy. However, CD28/B7 remains the potent pathway to increase the releasing of IL12, and IFNα whereas IL12 plays a role in proliferation as well as T cell differentiation. When CD28/B7 induces bcl-xl, it affects the viability of cells' IFNα secretion functions to increase the expression of MHC Class I.[8,12–14]

$CD8^+$ T Lymphocytes has been proven to be anti-tumor effector cells which are phenotypically and functionally identical to cytotoxicity T cells (CTL). CD8+ T cells destroy the virus infected cells or tumor cells through perforin and granzyme that can induce apoptosis.[15,16] During the contact of CTL with cancer cell granzyme substance, serine protease TNFα can get into cytoplasm which induces necrosis. The interaction of CTL fas with cancer cell fas ligand results in apopototic death.[12,17] This study aimed to investigate the role of CD80, CD86, MHC Class I and CD8+ in advanced stage Grade 3 NPC.

Materials and Methods

We conducted an epidemiological study in NPC patients who visited Otolaryngology outpatient clinic of Dr. Moewardi Hospital, Surakarta, Central Java, Indonesia from January 2011 to December 2014. The data were obtained from the patients' medical records and histopathologic examination results. They were then classified demographically while the histopathologic findings were classified based on WHO Classification. The demographic characteristics included gender, age, job, education and histopathologic grade described by WHO.

Totally 32 samples were analyzed using cross-sectional design. These 32 samples underwent immunohistochemical analysis of WHO Type 3, which were then analyzed for LMP1, CD80, CD86, MHC-I and CD8 expressions. The diagnosis of NPC was confirmed with biopsy performed with local or general anesthesia using rigid or flexible endoscopy. The extent of the nasopharyngeal local tumor was evaluated with Computerized Tomography (CT) or Magnetic Resonance Imaging (MRI). The distant metastasis was diagnosed with chest X-ray, bone scintigraphy and abdominal ultrasonography. The clinical staging was constructed using American Joint Committee on Cancer (AJCC) staging system.

The resected samples were placed in phosphate buffer saline (PBS) pH 7.0 diluted in formalin 10% and delivered directly to pathology laboratory of Dr. Moewardi Hospital for hematoxylin eosin staining (HES) by a pathologist.

Histopathologic preparations of Grade 3 NPC were sectioned in 4 micron thicknesses and placed on poly-L-Lysine glass slide (SIGMA). Next, the antigen retrieval deparaffination was done using microwave oven with buffer citrate at a pH of 6.4, then the slide was soaked in methanol H_2O_2 0.3% for 30 minutes and then rinsed it in PBS.

Afterwards, humidified chamber was used, and blocking reagent was applied for 30 minutes and washed with PBS. In a series, primary antibody was added. These primary antibodies were monoclonal Rabbit anti-human LMP1 antibody (Santacruz Biotechnology-Inc), monoclonal Rabbit anti-human CD80 and CD86 antibody (Abcam), polyclonal Rabbit anti-human MHC-I antibody (Santacruz Biotechnology-Inc), and monoclonal Rabbit anti-human CD8+ (Biogenex). These were kept in the refrigerator for 18 hours and then washed with PBS; secondary universal antibody (Trecckie) labelled biotin was added at 30°C, and then washed with PBS. Next, diominobenzedine substrate was added and washed with PBS, staining with major counterstain hematoxylin and finally glass block was patched.

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Each slide is analyzed by an expert of Physiologic Division of Medical Faculty of Brawijaya University, by using immunorating. The data was analyzed statistically by using path analysis.

Results

Demographic characteristics of NPC patients

There were 434 patients with NPC. The demographic characteristics of the patients were documented including age, sex, job and histopathologic findings. The distribution of NPC in Surakarta and other surrounding districts (Sukoharjo, Karanganyar, Boyolali, Sragen, Wonogiri and Klaten) is presented in Table 1. Histopathologically, WHO Grade LMP1 NPC was the most common type of NPC in Surakarta and its surroundings (Table 1).

Most patients were males (74.42%), aged between 51 and 60 years (41.94%) with low education background (55.07%), and majority of patients worked as farmers (57.83%). Our histopathologic examination revealed that the most common type of NPC was Grade 3 (88.25%) followed by Grade 2 (9.68%) and Grade 1 (2.07%) (Table 2).

Table 2. The demographic characteristics of the patients

Characteristics		Total	Percentage
Age	21–30	16	3.69%
	31–40	58	13.36%
	41–50	93	21.43%
	51–60	182	41.94%
	> 60	85	19.59%
Gender	Man	323	74.42%
	Female	111	25.58%
Job	Private employees	36	8.29%
	Government employees	45	10.37%
	Entrepreneur	102	23.50%
	Farmer	251	57.83%
Education	Primary school	239	55.07%
	Junior high school	115	26.50%
	Senior High School	48	11.06%
	University	32	7.37%
Histopathology	Grade 1	9	2.07%
	Grade 2	42	9.68%
	Grade 3	383	88.25%

Table 1. The distribution of patients with nasopharyngeal carcinoma in Surakarta and surroundings between 2011 and 2014

Zone	Surakarta	Sukoharjo	Karang Anyar	Boyolali	Sragen	Wonogiri	Klaten
Total population	552.650	863.693	840.171	951.817	896.201	942.377	1.303.910
Man	273.038	428.159	424.597	468.693	444.003	458.090	646.335
Female	279.612	435.534	415.574	483.124	452.198	484.287	670.572
Patients with nasopharyngeal carcinoma	14 (3.2%)	114 (26.2%)	50 (11.5%)	67 (15.4%)	92 (21.1%)	62 (14.2%)	35 (8%)
Male	11	86	26	49	80	46	26
Female	3	28	24	18	12	16	9
Age of Patients with NPC							
21–30	1 (7.1%)	5 (4.3%)	2 (4%)	3 (4.4%)	3 (3.2%)	1 (1.6%)	1 (2.8%)
31–40	7 (50%)	10 (8.7%)	4 (8%)	7 (10.4%)	17 (18.4%)	7 (11.3%)	6 (17.1%)
41–50	2 (14.3%)	25 (22%)	7 (14%)	16 (23.8%)	18 (19.5%)	21 (33.8%)	5 (14.2%)
51–60	2 (14.3%)	58 (50.8%)	17 (34%)	33 (49.2%)	18 (19.5%)	39 (62.9%)	15 (42.8%)
> 60	2 (14.3%)	15 (13.1%)	13 (26%)	11 (16.4%)	16 (17.3%)	20 (32.2%)	8 (22.8%)
Job							
Private employees	2 (14.2%)	7 (6.1%)	5 (10%)	7 (10.4%)	5 (5.4%)	7 (11.3%)	3 (8.5%)
Government employees	2 (14.2%)	11 (9.6%)	4 (8%)	8 (11.9%)	6 (6.5%)	8 (12.9%)	6 (17.1%)
Entrepreneur	9 (64.2%)	22 (19.2%)	9 (18%)	16 (23.8%)	22 (23.9%)	15 (24.2%)	9 (25.7%)
Farmer	1 (7.1%)	72 (63.1%)	31 (62%)	36 (53.7%)	39 (42.4%)	53 (85.4%)	17 (48.5%)
Education							
Primary school	1 (7%)	72 (63.1%)	33 (66%)	37 (55.2%)	49 (53.2%)	31 (50%)	16 (45.7%)
Junior high school	8 (57.1%)	26 (22.8%)	8 (16%)	16 (23.8%)	31 (33.6%)	16 (25.8%)	10 (28.5%)
Senior High School	4 (28.5%)	11 (9.6%)	4 (8%)	8 (11.9%)	7 (7.6%)	8 (12.9%)	6 (17.2%)
University	1 (7%)	5 (4.3%)	5 (10%)	6 (8.9%)	5 (5.4%)	7 (11.2%)	3 (8.5%)
Grade							
Grade 1	0 (0%)	1 (0.87%)	1 (2%)	1 (1.4%)	3 (3.2%)	1 (1.6%)	2 (5.7%)
Grade 2	2 (14.2%)	12 (10.5%)	7 (14%)	10 (14.9%)	4 (4.3%)	5 (8%)	2 (5.7%)
Grade 3	12 (85.7%)	101 (88.5%)	42 (84%)	56 (83.5%)	85 (92.4%)	56 (90.3%)	31 (88.5%)

(NPC: Nasopharyngeal carcinoma)

Our study revealed that there was no significant association among tested variables in Stage 3-4 Grade 3 nasopharyngeal tumors. It demonstrates that variables were homogeneous.

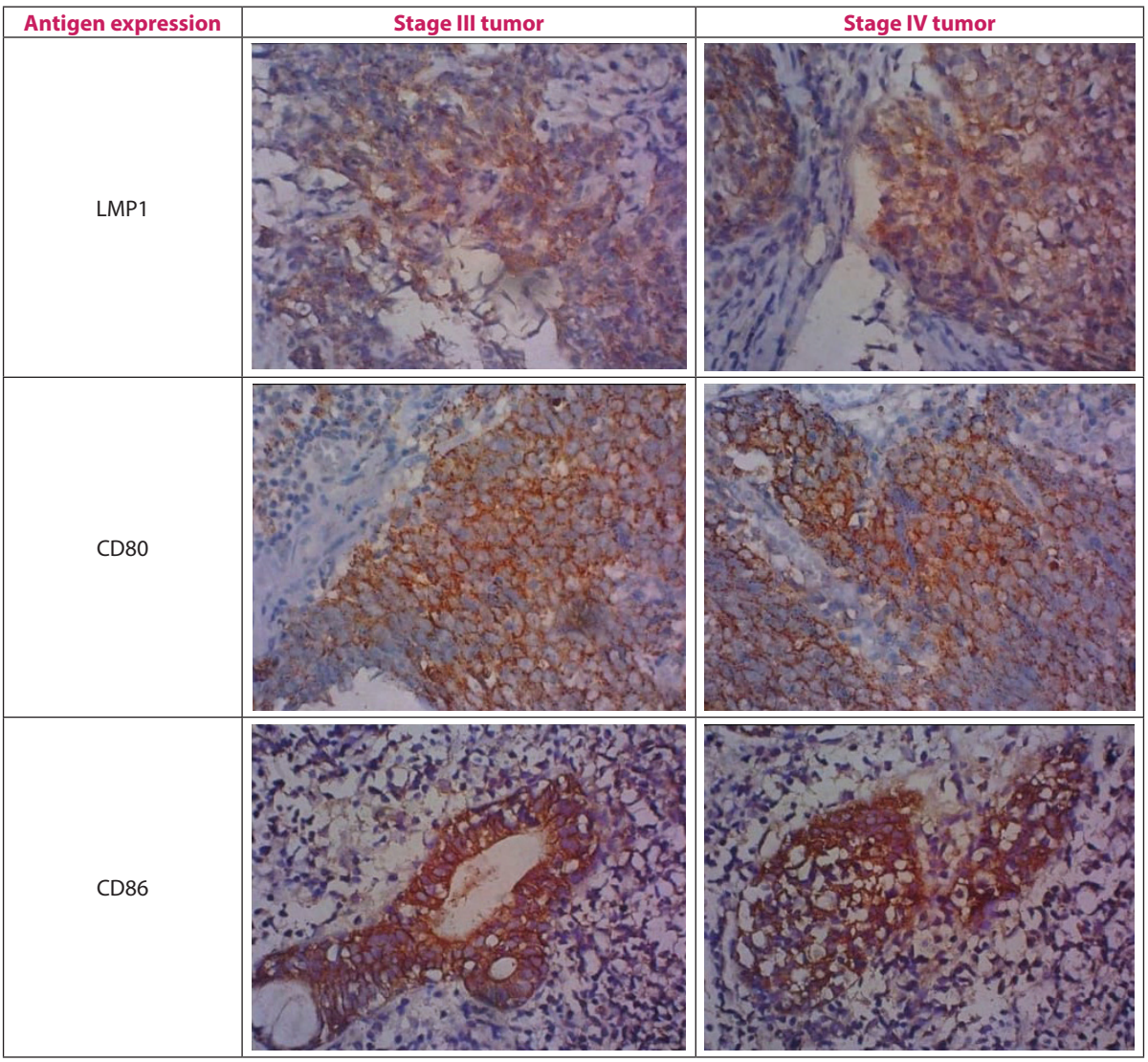
Immunohistochemistry staining (IHC) (Fig. 1)

Path analysis

We found a statistically significant correlation between LMP1 and CD86 expression in patients ($p<0.0001$). However, no statistically significant correlation was found between LMP and CD80 ($p=0.110$). Similarly, there was a correlation between LMP and MHC Class I expression ($p=0.012$)(Table 4).

Table 3. The stage distribution and antigen expression of Grade 3 nasopharyngeal carcinoma

Group Statistics					
	Stage	N	Mean	Std. deviation	p-value
LMP1	Stage III	16	41.5000	24.62711	0.515
	Stage IV	16	47.6594	28.14587	
CD80	Stage III	16	46.7988	31.86187	0.755
	Stage IV	16	50.2500	30.08967	
CD86	Stage III	16	56.5263	18.19714	0.608
	Stage IV	16	52.3275	26.81301	
MHC1	Stage III	16	65.9906	10.98441	0.451
	Stage IV	16	68.9162	10.67543	
CD8	Stage III	16	31.6875	21.45519	0.574
	Stage IV	16	27.4875	20.29272	



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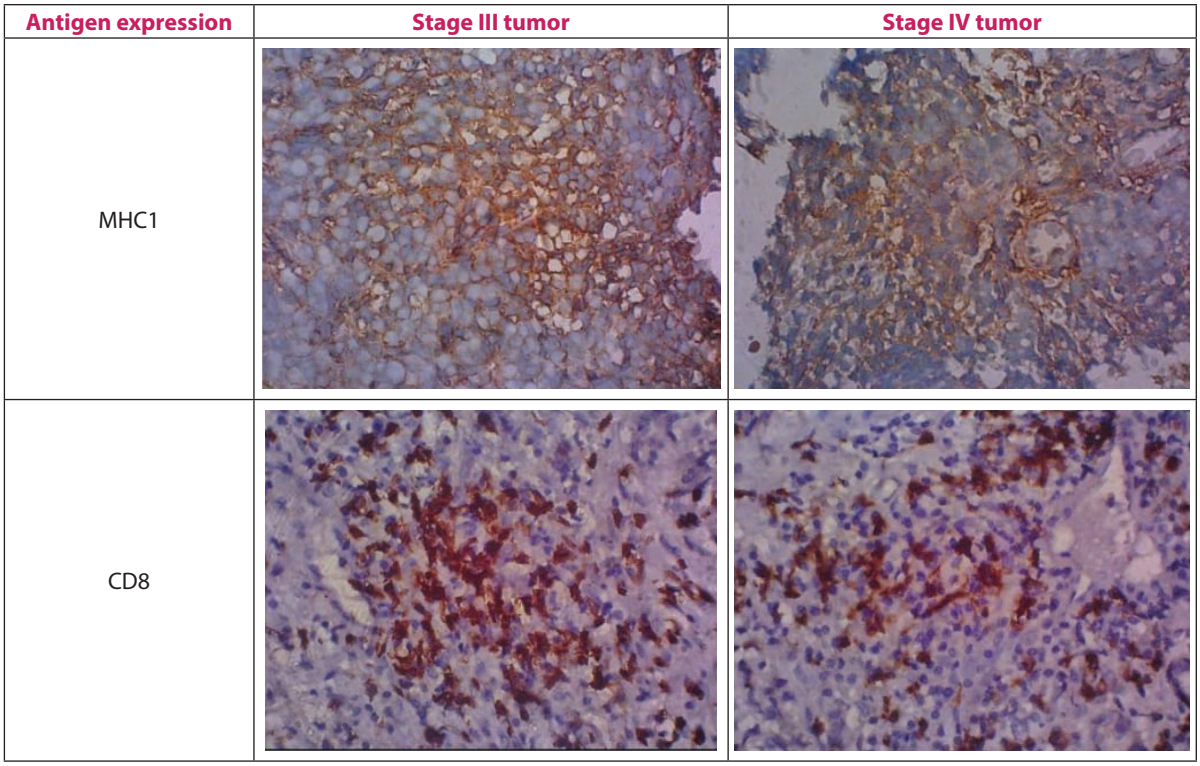

Figure 1. Expression of LMP1, CD80, CD86, MHC-I and CD8 in nasopharyngeal carcinoma (NPC) stage III and IV tumors. Fraction of cells with positive staining was recorded. Section were immunostained as was described in the Material and Methods. Bar 4 - 5μM.

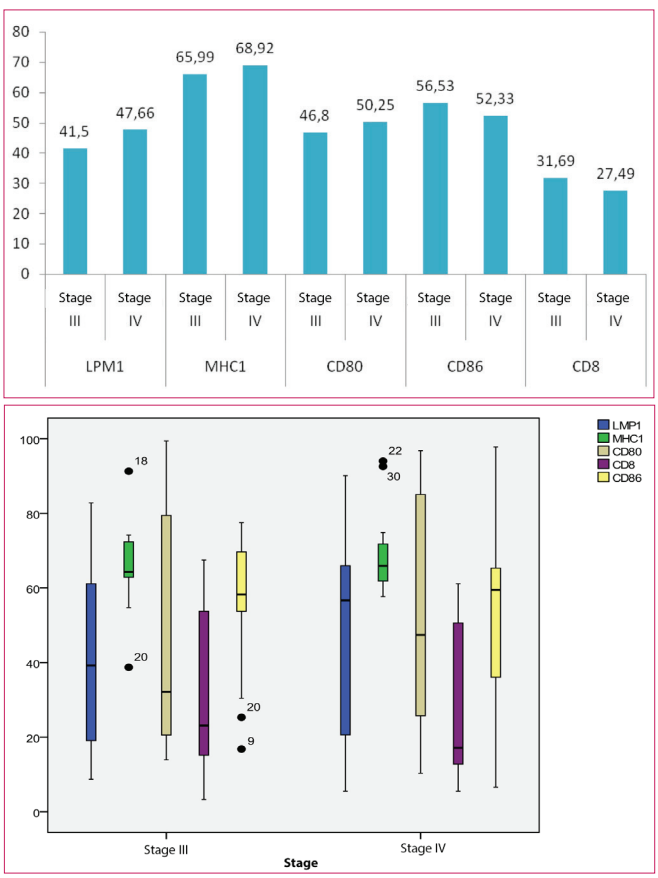

Table 4. Regression table

			Estimate	Standard error	C.R.	P
CD80	←	LMP1	-0.322	0.201	-1.598	0.110
CD86	←	LMP1	-0.499	0.134	-3.721	***
CD86	←	CD80	-0.104	0.115	-0.903	0.366
MHC1	←	LMP1	-0.152	0.078	-1.938	0.053
MHC1	←	CD86	0.060	0.090	0.664	0.507
CD8	←	LMP1	-0.090	0.152	-0.589	0.556
CD8	←	MHC1	-0.789	0.315	-2.509	0.012
CD8	←	CD80	0.004	0.104	0.036	0.971
CD8	←	CD86	-0.342	0.162	-2.115	0.034

MHC1 : Major histocompatibility complex class I
LMP1 : Latent membrane protein-1

Figure 2. Expression of LMP1, CD80, CD86, MHC-I and CD8 in Stage III and IV nasopharyngeal carcinoma (NPC).

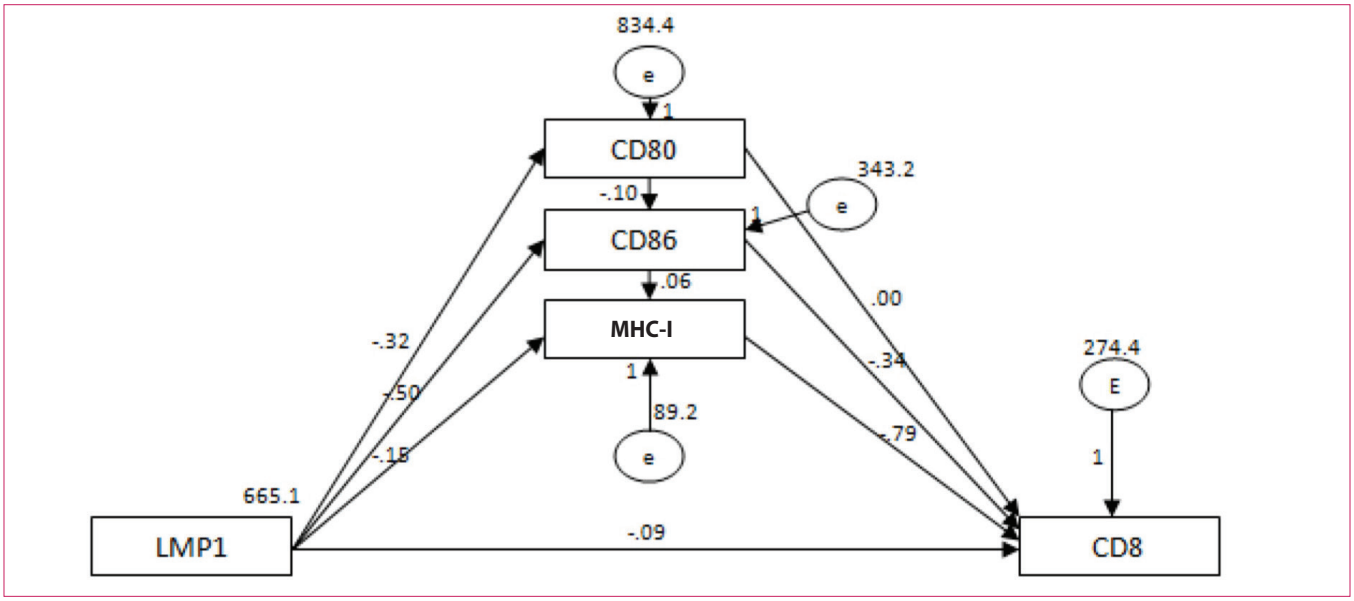

Figure 3. Path analysis of the expression LMP1, CD80, CD86, MHC-I and CD8 in stage III and IV nasopharyngeal carcinoma (NPC) shows that there is no correlation between CD8 and MP expression in terms of CD80 expression (p = 0.110), while a statistically significant correlation was found between CD8 and LMP through CD86 expression(p<0.0001) and LMP1 to $CD8^+$ through MHC 1 (p = 0.0114).

Discussion

The demographic data of NPC patients demonstrates that NPC is more common in males than in females. This finding may be related to the smoking habits and the job s of the patients that may be related to contact with carcinogenic agent. In our study, most of the subjects were farmers. This occupation predisposes them to cancer since they are often exposed to pesticides without applying a safe procedure while using it.

The most common affected age is between 51 and 60 years. This may be due the long process of carcinogenesis and decreased immune system. Low education background also may play a role in delayed awareness of this cancer, since the early symptom of NPC is quite similar to those of upper respiratory tract infection and NPC is anatomically hidden. That is why we were interested in investigating the host immune system in advanced stage Grade 3 NPC.

Our molecular study of 32 samples of Grade 3 NPC revealed that there was no direct correlation between LMP1 and CD8 (p=0.556). The relationship was similar to the correlation between LMP1 and CD80. However, we found that LMP1 and CD86 seems to be associated with MHC Class I (p=0.012) as well.

This study also showed that the relationship between LMP1 and CD8 was mediated by CD80 (p=0.276), and that was a lower association than that of between LMP1 and CD8 (-0.114). This demonstrates that the relationship between LMP1 and CD8 could be through CD80 (p=0.110).

CD8 expression was found to be lower in the tumor tissue which may indicate a lower activity of CD8+ T lymphocytes (immunosuppression). LMP1 might decrease CD8+ cells' activity through CD80 signaling.[10,12,14]

The relationship between LMP1 and CD8 through CD86 (p=0.539) was found to be weaker than that between LMP1 and CD8 (p=-0.114). However, LMP1 was found to be associated with CD8 through CD86 (p<0.0001). This demonstrates that tumor eliminating cytotoxic CD8+ cells can be inhibited through CD86.[7,9,11]

The association between LMP and MHC Class I (p=0437) is less prominent compared with the association between LMP1 and CD8 (0.114). This finding is probably due to the decreased but statistically significant association of LMP1 and CD8 through MHC Class I (p=0.012). It has been suggested that LMP1 could decrease the antigen recognition of CD8+ T Lymphocyte through MHC

Class I.[7–9,13,14,18] The effect of LMP1 on CD8+ T cells could be mediated by CD86. Increased LMP1 expression was found to be associated with decreased MHC Class I expression that leads to decreased CD8 expression. These findings suggest immunsuppressive effects of NPC cells.

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Declaration of conflicting interests
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