Exp Oncol 2006
28, 1, 40–43

Experimental ONCOLOGY

THE ASSOCIATION BETWEEN CD99 AND LMP-1 EXPRESSION IN NASOPHARYNGEAL CARCINOMA

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Aim: **To characterize the roles of LMP-1 and CD99 in nasopharyngeal carcinogenesis, we undertook this pilot study of LMP-1 and CD99 expressions in nasopharyngeal cancer (NPC).** ***Materials and Method:*** **40 NPC tissue samples were grouped according to the WHO classification. Immunohistochemical studies were performed using monoclonal antibodies against EBV latent membrane protein 1 (LMP-1) and CD99 protein. In addition, CD99 expression was evaluated in 10 samples of non-neoplastic nasopharyngeal epithelium.** ***Results:*** **LMP-1 was detected in 12 of the 40 (30.0%) cases and its expression was found to be confined to epithelial tumor cells. WHO type I NPC samples were completely negative for LMP-1, whereas WHO type III NPC samples showed highest expression. Interestingly, CD99 was expressed in all of the non-neoplastic nasopharyngeal epithelium samples along the cytoplasmic border. CD99 expression was noted in NPC tumor cells (5 of the 40 cases, 12.5%) and in surrounding lymphoid stroma (23 of the 40 cases, 57.5%), but was not expressed in WHO type I NPC. In the 12 LMP-1 positive cases, 9 cases (75.0%) were CD99 negative, and 3 cases (25.5%) were CD99 positive. There was a statistical significance between LMP-1 and CD99 expression in lymphoid stroma.** ***Conclusion:*** **Our results suggest that the LMP-1 induced down-regulation of the CD99 pathway is important in nasopharyngeal carcinogenesis, and that the expression of CD99 in lymphoid stroma may regulate immune response to NPC.**
Key Words: **Epstein—Barr virus, latent membrane protein-1, CD99, nasopharyngeal carcinoma, Hodgkin lymphoma.**

The Epstein-Barr virus (EBV) is a γ herpes virus that infects more than 90% of the human population, although the majority of carriers remain asymptomatic. EBV infection is associated with nasopharyngeal carcinoma (NPC) and Hodgkin lymphoma (HL) [1].

The WHO classification of nasopharyngeal carcinomas distinguishes two major types, i.e., squamous cell carcinoma and non-keratinizing carcinoma, the latter of which includes undifferentiated carcinoma [2].Although the association between non-keratinizing NPC (particularly of the undifferentiated subtype) and EBV is well established, NPC oncogenesis is not a simply consequence of EBV infection.

LMP-1 is the major transforming protein of EBV and behaves as a classical oncogene. LMP-1 is an integral membrane protein of 63 kDa, and can be subdivided into three domains [3]. Its two distinct functional domains, referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2), were identified because of their ability to activate the NF-κB transcription factor pathway [3].

The consequences of NF-κB activation are numerous and include the upregulation of anti-apoptotic gene products and the downregulation of cell surface molecules CD99. In NPC cells, LMP-1 induces TRAF1 expression and promotes its anti-apoptosis activity via the NF-κB signaling pathway, and thus suppresses apoptosis in NPC cells [4].

CD99 is a strongly sialoglycosylated 32 kDa transmembrane protein [5] and is involved in multifactorial cellular events, such as cell-cell adhesion during hematopoietic cell differentiation, the apoptosis of immature thymocytes and neuronal cells, and T-cell activation [6, 7]. NPC and Hodgkin lymphoma, especially Reed—Sternberg cells (HRS) share several features that warrant closer examination [8]. Kim et al. [9] reported that HRS cells from the lymph nodes of HL patients, consistently lack CD99 surface expression. Moreover, induced LMP-1 expression has been demonstrated to directly down-regulate CD99 in B-cell lines at the transcriptional level via the NF-κB pathway [10, 11]. This finding possibly indicates a role for the LMP-1 induced down regulation of CD99 in the generation of HRS-like cells. By comparison, little is known about NPC in this respect.

The aim of this study was to evaluate the expressions of LMP-1 and CD99 in NPC and to search for correlations between these expressions and tumor growth.

MATERIALS AND METHODS

Patients and specimens. A review of the H&E sections of 40 patients revealed 5 squamous cell carcinomas (SCCs, WHO type I), 20 non-keratinizing carcinomas (NKCs, WHO type II), and 15 undifferentiated carcinomas (UCs, WHO type III). There were 28 men and 12 women (M : W=2.3 : 1), and patient ages ranged from 18 to 80 years (mean age 52.1 years). NPC diagnoses were confirmed by nasopharyngeal biopsy at the Department of Pathology, Chonnam National University Hospital.

Immunohistochemistry. We reviewed the hematoxylin and eosin stained slides of all cases, and then selected well preserved tissue blocks of formalin-fixed, paraffin-embedded samples. The immunohistochemical analysis included monoclonal antibody for LMP-1 (DAKO, clones CS1-4, CA, USA: dilution 1 : 400), and CD99 (DAKO, clones 12E7, CA, USA: dilution 1 : 50). Immunostaining was performed using the routine avidin-

Received: November 28, 2005.
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Abbreviations used: EBV – Epstein–Barr virus; LMP-1 – latent membrane protein-1; HL – Hodgkin lymphoma; HRS cell – Hodgkin Reed–Sternberg cell; NPC – nasopharyngeal carcinoma.

biotin complex (ABC) method. Briefly, representative paraffin blocks were consecutively sectioned at 4μm and immunostainusing a Microprobe Immuno/DNA stainer (Fisher Scientific, CA, USA). Sections were deparaffinized in xylene, and treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogeneous peroxidase activity. For LMP-1 and CD99 immunostaining, sections were microwaved in citrate-phosphate buffer (pH 6.0) once, and then incubated with antibodies at room-temperature for 1 h. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody to detect primary antibodies, and was applied by incubation for 7 min at 45 °C. The streptavidin-horseradish peroxidase (Research Genetics, USA) detection system used to visualize capillary channels, by incubation for 10 min at 45 °C. After drainage, tissue sections were reacted with 0.02% diaminobenzidine as chromogen. They were then counterstained with hematoxylin and mounted in Universal Mount medium (Research Genetics, USA). Cortical thymus tissue was used as positive control for CD99 staining. Negative controls were treated similarly with the exception of primary antibodies.

Evaluation of immunohistochemical results. All immunostained slides were evaluated independently by two observers, unaware of background information, who both performed evaluations in duplicate. Appropriate positive and negative controls were prepared in the same fashion. Cytoplasmic and/or membranous staining patterns were considered positive for LMP-1. In addition, staining in the Golgi region was also considered positive for LMP-1. However, for CD99 only membranous staining was considered positive. Positive cell frequencies were evaluated semi-quantitatively and are expressed as ratios of positive-stained tumor cells versus the total tumor cell population. They were graded from 0 to +3 as follows: 0 = no tumor cells, +1 = less than 25% tumor cells, +2 = 25% to 50% tumor cells, and +3 = greater than 50% tumor cells. The intensity of staining was not graded, and any expression of LMP-1 or CD99 was classified as positive.

Statistical analysis. Statistical analysis was performed using SPSS for Windows, version 10.0.1 (SPSS Inc, USA). Results were statistically evaluated using the Chi-square test or the Mann Whitney-*U* test, as modified by Yates. A significance level of 0.05 was used for all statistical tests.

RESULTS

LMP-1 expression in NPC samples. In order to identify EBV-encoded LMP-1 expression in NPC, immunostaining for LMP-1 was performed using CS1-4 monoclonal antibody by the ABC method. A dark red color in the cell membrane and cytoplasm, especially in Golgi areas, was considered as a positive reaction. LMP-1 was detected only in tumor cells; infiltrating lymphocytes and stroma were unstained (Fig. 1). Moreover, staining intensities varied from cell to cell. In infiltrative lymphocytes and stroma, LMP-1 was completely negative. LMP-1 expression was most frequently positive in WHO type III than in WHO types I and II, and this type-associated difference was significant ($p = 0.034$, Table 1).

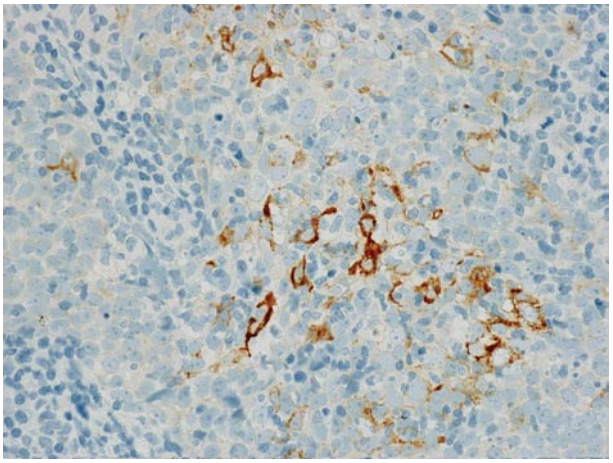

Fig. 1. Immunoreactivity for LMP-1 in WHO type II nasopharyngeal carcinoma. Tumor cells exhibited cytoplasm and Golgi apparatus focally positive immunoreactivity. The lymphocytes surrounding the tumor cells were negative (LMP-1, X 400)

Table 1. Distribution of LMP-1 expression in CD99 in lymphoid stroma/tumor tissue according to the positive frequency

Localization of expression	LMP-1				*p* value
	Grade	Negative	Positive	Total	
CD99 in lymphoid stroma	Negative	14	3	17	0.021
	Positive	14	9	23	
	Total	28	12	40	
CD99 in epithelial tumor cells	Negative	26	9	35	0.294
	Positive	2	3	5	
	Total	28	12	40	

Table 2. Distribution of LMP-1, CD99, and bcl-2 expression according to the histologic subtypes

Expression of the marker	SCC	NKC	UC	Total	p value
LMP-1	0/5	5/20	7/15	12/40	0.034
CD99 in lymphoid stroma	0/5	11/20	11/15	23/40	0.440
CD99 in tumor	0/5	4/20	1/15	5/40	0.720

Notes: SCC – squamous cell carcinoma, WHO type I; NKC – non-keratinizing carcinoma, WHO type II; UC– undifferentiated carcinoma, WHO type III

CD99 expression in non-neoplastic nasopharyngeal epithelium and NPC samples. Initially, we evaluated CD99 immunostaining in the 10 non-neoplastic nasopharyngeal tissue samples. Some of these samples were obtained from anterior and posterior nasopharyngeal walls lined by stratified squamous epithelium, whereas others were from posterior nares or the roof of the posterior wall lined with ciliated respiratory-type columnar epithelium. All samples showed CD99 expression along nasopharyngeal epithelial cytoplasmic borders and in some lymphocytes in adjacent areas (Fig. 2).

We then evaluated CD99 immunostaining in the NPC samples, where CD99 was more frequently expressed in surrounding lymphoid stroma (23 of 40 cases, 57.5%, Fig. 3, *a*, Table 1) than in epithelial tumor cells (5 of 40 cases, 12.5%, Fig. 3, *b*). According to WHO types, CD99 expression in lymphoid stroma was highest in WHO type III (11 of 15 cases, 73.3%), and was completely negative in WHO type I. Moreover, the staining intensity of CD99 was stronger in infiltrating lymphocytes and stroma than in tumor cells. No evident correlation was observed between CD99 expression in stroma or tumor cells and WHO histologic classification (Table 1). However, a significant positive correlation was observed between LMP-1 and CD99 expressions in lymphoid stroma (Pearson correlation

coefficient = 0.298, p = 0.021, Table 2). And, in patients with positive CD99 expression in epithelial tumor cells (5 of the 40 cases), LMP-1 was negative in 2 cases and positive in 3 cases, whereas in patients with positive LMP-1 expression, CD99 was negative in 9 of 12 cases (75.0%), and positive in 3 of 12 cases (25.5%).

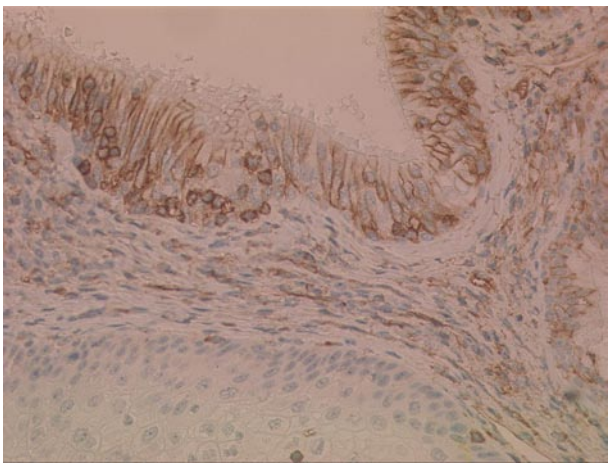

Fig. 2. CD99 expression in non-neoplastic nasopharyngeal mucosa. All cases showed CD99 expression along the cytoplasmic borders of nasopharyngeal epithelium and in some lymphocytes and vascular endothelial cells in adjacent areas

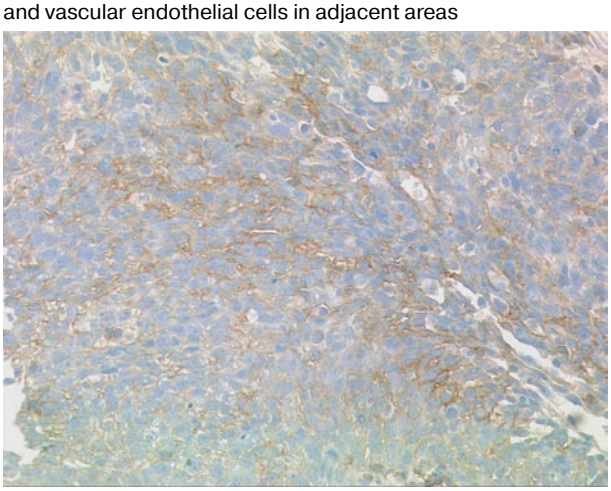

Fig. 3, *a*. Strong CD99 immunostaining in WHO type III nasopharyngeal carcinoma. This pattern is similar to that shown by Ewing's sarcoma and primitive neuroectodermal tumor, which also show membranous accentuation (CD99, X 400)

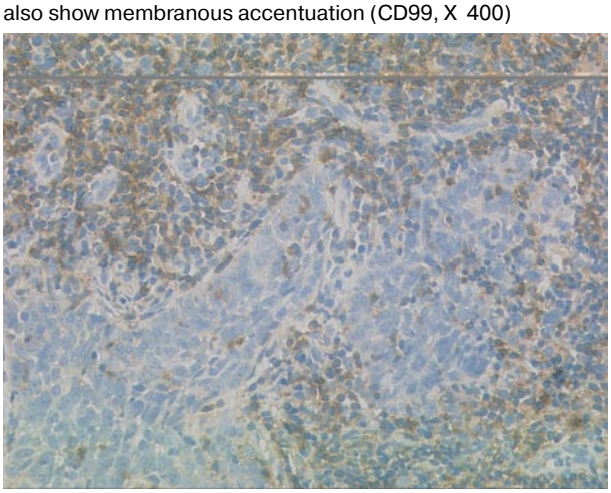

Fig. 3, *b*. Immunoreactivity for CD99 in WHO type II nasopharyngeal carcinoma. Tumor cells exhibited negative immunoreactivity, but lymphocytes surrounding tumor nests were strongly positive (CD99, × 400)

DISCUSSION

LMP-1 has a significant effect on epithelial cell growth, and it inhibits the differentiation and induces the morphologic transformation of some cell lines. In this study, in order to identify EBV-encoded LMP-1 expression in NPC, immunostaining of LMP-1 was performed using CS1-4 monoclonal antibody by the ABC method. Of the 40 NPC samples, 12 (30.0%) showed LMP-1 expression confined to epithelial tumor cells, which is consistent with the observation that viral DNA is detectable in malignant epithelial cells rather than in the abundant infiltrating lymphoid cells [12]. LMP-1 has been detected in less than 50% of NPC using several different monoclonal antibodies [13, 14]. However, these antibodies did not recognize all forms of LMP-1, thus the reported rate of LMP detection may represent an underestimation [15]. Moreover, it is possible that in some tumors LMP-1 is expressed at below the level of detection or that as a cancer progresses the expression of viral protein is no longer required as other genetic changes substitute for the effects of viral proteins [15].

Kim et al. [9] reported that HRS cells from the lymph nodes of HL patients consistently lack of CD99 surface expression, and enhanced LMP-1 expression has been shown to directly down-regulate CD99 in B cell lines at the transcriptional level via the NF-κB pathway. In contrast, in the present study, LMP-1 was found to be positive in 3 of 5 NPC samples expressing CD99. In terms of LMP-1 immunopositivity, of the 12 LMP-1 positive NPC samples, CD99 was negative in 9 cases (75.0%) and positive in 3 (25.0%). Our findings, and those of previous reports [9], indicate that LMP-1 may induce the down-regulation of CD99 and play an important role in nasopharyngeal carcinogenesis. In view of the finding that CD99-independent LMP-1 induced pathways generate H-RS cells in a synergistic way [16], our finding of LMP-1+CD99+ NPC cells indicates the need for further studies to elucidate the role of CD99 in NPC carcinogenesis.

Infiltrating lymphocytes and lymphoid stroma are characteristic histopathologic features of WHO type III NPC. Lymphoid stroma is predominantly composed of T cells, and CD4 and CD8 expressing cells in variable proportions [17]. In the present study, a considerable number of surrounding lymphocytes showed strong reactivity for CD99, and a significant positive correlation was observed between LMP-1 expression in tumor cells and CD99 expression in lymphoid stroma (Pearson correlation coefficient = 0.298, p = 0.021, Table 2). CD99 is present on all human T- cells, but has no clear biologic function [5, 18]. Studies on mature peripheral T cells have shown that co-ligation of CD99 and CD3 may enhance the expression of CD25, CD69, and CD40 ligand, induce T-cell proliferation, and also promote the production of TNF-α and IFN-γ [7]. These findings suggest that CD99 may contribute to T-cell activation in NPC microenvironment and promote communication between tumor cells and tumor-infiltrating T lymphocytes.

In conclusion, our results suggest that LMP-1 induces the down-regulation of CD99, and that this might be an important step in nasopharyngeal carcinogenesis, as shown in the HRS synthesis in HL. Moreover, our findings show that CD99 expression in lymphoid stroma is influenced by the presence of LMP-1.

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ВЗАИМОСВЯЗЬ МЕЖДУ ЭКСПРЕССИЕЙ CD99 И LMP-1 В КАРЦИНОМЕ НОСОГЛОТКИ

Цель: **изучить роль экспрессии латентного мембранного белка-1 (LMP-1) EBV и CD99 при развитии карциномы носоглотки (КНГ).** ***Материалы и методы:*** **в соответствии с классификацией ВОЗ 40 образцов КНГ были отнесены к типам I, II, III. Иммуногистохимический анализ проводили с использованием моноклональных антител против LMP-1 и CD99. Уровень экспрессии CD99 также был определен в 10 образцах нетрансформированного эпителия носоглотки.** ***Результаты:*** **экспрессия белка LMP-1 была выявлена в 12 из 40 образцов КНГ (30%) только в эпителиальных клетках опухоли. В КНГ I типа все образцы были негативными по экспрессии LMP-1, а в КНГ III типа была обнаружена самая высокая степень экспрессии. Интересно, что экспрессия CD99 была выявлена во всех контрольных образцах эпителия носоглотки по краю цитоплазмы. Ее также наблюдали в опухолевых клетках (в 12,5 образцов КНГ) и в окружающей лимфоидной строме (57,5% образцов КНГ), но не выявляли в КНГ I типа. Среди 12 LMP-1 позитивных образцов было выявлено 9 CD99 негативных (75,%) и 3 — CD99 позитивных (25,5%). Также наблюдались статистически значимые различия между экспрессией LMP-1 и CD99 в лимфоидной строме.** ***Выводы:*** **LMP-1 индуцирует подавление каскада CD99, выполняющего важную роль в образовании опухолей носоглотки. Экспрессия CD99 в лимфоидной строме может регулировать иммунную реакцию на КНГ.**

Ключевые слова: **вирус Эпштейна—Барр, латентный мембранный белок-1, CD99, карцинома носоглотки, лимфома Ходжкина.**