

Epstein-Barr Virus-encoded Latent Membrane Protein 1 Co-expresses with Epidermal Growth Factor Receptor in Nasopharyngeal Carcinoma

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Latent membrane protein 1 (LMP-1) is the only Epstein-Barr virus (EBV)-encoded oncogenic protein that has been detected in nasopharyngeal carcinoma (NPC), a cancer that is closely associated with EBV. Previous *in-vitro* studies have demonstrated that LMP-1 can upregulate epidermal growth factor receptor (EGFR) in epithelial cells. It was not established whether this cellular effect exists in NPC. To assess the association between LMP-1 and EGFR in NPC tissues, 60 NPC specimens were examined by immunohistochemistry using anti-LMP-1 antibody (CS 1-4) and anti-EGFR antibodies (EGFR 1, EGFR 1005). The results revealed that 41 (68.3%) specimens were immunopositive for LMP-1 and 44 (73.3%) specimens over-expressed EGFR. Morphologically, the expressions of LMP-1 and EGFR were homogeneously distributed in the tumor nests. In addition, the correlation between LMP-1 and EGFR was statistically significant ($P < 0.001$, χ^2 test, d.f.=1). To elucidate further the correlation between LMP-1 and EGFR *in vivo* and *in situ*, an indirect dual immunofluorescence assay was conducted, using secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or indocarbocyanine (Cy3). The results disclosed an intimate co-expression of LMP-1 and EGFR. In summary, the data indicate that over-expression of EGFR is a common phenomenon in NPC, and that EGFR is co-expressed with LMP-1 in NPC. Thus, EBV may play a role in the tumorigenesis of NPC through the effects of LMP-1 and EGFR.

Key words: Immunohistochemistry — Indirect dual immunofluorescence — Co-expression — Epidermal growth factor receptor — Latent membrane protein 1

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck cancers in Taiwan. The annual incidence of NPC in men was estimated at 6.74 per 100,000 in 1994; men are two to three times more vulnerable than women.¹⁾ The mean age of the victims is around 45 years. This is about 10 years younger than the mean age of victims of other head and neck cancers. The pathogenesis of NPC is still obscure, but the proposed etiologic factors include genetic inheritance, environmental carcinogens, and Epstein-Barr virus (EBV).

In 1966, Old *et al.* first demonstrated elevated anti-EBV antibody titers in patients with NPC.²⁾ Subsequently, by using DNA hybridization, zur Hausen *et al.* and Wolf *et al.* proved that NPC tumors harbor the EBV genome.^{3,4)} More recently, sensitive methods, such as polymerase chain reaction and *in-situ* hybridization probing by EBV-encoded small RNA (EBER), have established that EBV is present in nearly all NPC tumors irrespective of histologic differentiation or geographic distribution.⁵⁻⁷⁾

Oncologists are very interested in the role played by EBV in the pathogenesis of NPC because EBV is consid-

ered a potentially oncogenic virus in humans. At least 4 EBV-encoded proteins are required to activate and immortalize infected B lymphocytes to lymphoblastoid cell lines (LCLs) *in vitro*, these proteins being EBV nuclear antigen 2 (EBNA 2), EBNA 3A, EBNA 3C and latent membrane protein 1 (LMP-1).⁸⁻¹⁰⁾ However, LMP-1 is the only EBV-encoded transforming protein found in EBV-associated non-B-cell malignancies; this protein was detected in about 65% of NPC specimens.^{6,11-14)} Biologically, LMP-1, encoded by the BNLF-1 open reading frame of the EBV genome, is a 63 kDa membrane protein containing six transmembrane domains.¹⁵⁾ Several studies showed that LMP-1 could affect the differentiation and growth of rodent fibroblasts and human epithelial cell lines.¹⁶⁻¹⁹⁾ Several cellular events mediated by LMP-1 were demonstrated, such as activation of anti-apoptotic A20 protein in B cells,²⁰⁾ transactivation of p53 and upregulation of bcl-2 in B cells through the activation of nuclear factor κ B (NF- κ B),^{21,22)} and upregulation of epidermal growth factor receptor (EGFR) in epithelial cells via tumor necrosis factor receptor-associated factors (TRAFs).²³⁾

Recent studies have characterized the structure and functions of peptide growth factors and their receptors.²⁴⁻²⁶⁾ After binding with extracellular ligands, the receptors,

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which are located on the cell membrane, autophosphorylate cytoplasmic tyrosine and activate a cascade of intracellular signal transduction pathways to evoke cell growth. However, the over-expression or mutation of these growth factors or receptors may contribute to the initiation and progression of cancer.²⁷⁾ Hendler and Ozanne first demonstrated the over-expression of EGFR in lung cancer.²⁸⁾ Subsequently, it was shown that EGF and EGFR played a very important role in the carcinogenesis of several other human epithelial malignancies, such as head and neck cancers.^{29,30)} As for NPC, Zheng *et al.* demonstrated the strong expression of EGFR in most NPC specimens by immunohistochemistry, and there seemed to be an association between the expression of LMP-1 and that of EGFR.¹⁴⁾ *In vitro*, Miller *et al.* demonstrated that LMP-1 could upregulate the expression of EGFR by immunoblotting assay.³¹⁾ These previous studies did not determine whether LMP-1 upregulates EGFR in NPC.

To elucidate the association between LMP-1 and EGFR in NPC *in vivo* and *in situ*, this study investigated 60 NPC specimens using immunohistochemistry and indirect dual immunofluorescence assays.

MATERIALS AND METHODS

Tissue acquisition Sixty fresh-frozen NPC biopsy specimens were supplied by the Department of Otolaryngology, National Taiwan University. After having been harvested from patients, these biopsy specimens were immersed in a 30% sucrose solution at 4°C for 12 h, embedded in Cryomatrix (Shandon, Life Science International Ltd., Cheshire, England), and stored in a deep-frozen state until sectioned.

Immunohistochemical detection of LMP-1 and EGFR The primary antibodies used in this study were mouse anti-LMP-1 monoclonal antibody (CS 1-4, DAKO, Copenhagen, Denmark),¹⁵⁾ mouse anti-EGFR monoclonal antibody (EGFR 1, DAKO),³²⁾ and rabbit anti-EGFR polyclonal antibody (EGFR 1005, Santa Cruz Biotechnology, Santa Cruz, CA). Before immunostaining, the specificity of the antibodies CS 1-4 and EGFR1 was confirmed by immunoblotting analysis using an LMP-1 transfected cell line and A431 cell line.

The Cryomatrix-embedded tissue blocks were sectioned with a thickness of 6 μm . Tissue sections were fixed at -20°C for 3 to 5 min. The fixation conditions for each primary antibody are listed in Table I. The immunostaining procedure included adequate hydration with Tris-buffered saline (TBS, pH=7.4) for 15 min, and blocking with 3% non-immunized goat serum (NGS) for 30 min. After blotting of NGS, the slides were incubated with a primary antibody (the conditions for each immunostaining are listed in Table I). Then the slides were incubated with a biotinylated secondary antibody at room temperature for 30 min. The incubation continued with horseradish peroxidase-conjugated avidin-biotin complex (ABC) or alkaline phosphatase (AP)-conjugated streptavidin (both purchased from DAKO), at room temperature for 30 min. The slides were thoroughly washed with TBS for 15 min at room temperature during each change of antibody. In the immunostaining of LMP-1 by the AP method, new Fuchsin (DAKO) was used as the chromogen: the color red represents a positive reaction. The standard ABC method was used to immunostain EGFR, and the tissue sections were additionally treated with 3% H_2O_2 in methanol for 30 min to eliminate the activity of endogenous peroxidase before blocking with NGS. Diaminobenzidine (DAB) (DAKO) was used as the chromogen in the ABC method: brown represents a positive reaction. Appropriate external positive control specimens, such as B95-8 cell smear, skin, and oral cancer, were stained simultaneously. A specimen of oral cancer, known to over-express EGFR, also served as a control for over-expression of EGFR. Normal respiratory epithelium on NPC specimens, whenever available, also served as an internal positive control for EGFR. Staining of these positive-control specimens, omitting the primary antibody, was used as a negative control. All the slides were counterstained with hematoxylin and were examined under light microscopy.

In this study, the results of the staining were interpreted by the first author (Dr. Sheen) and the pathologist (Dr. Chang). The expression of EBV-encoded LMP-1 was graded as positive or negative based on the presence of the red color of new Fuchsin (qualitatively). The over-expression of EGFR was interpreted as positive or negative based on the comparison between the staining of the nor-

Table I. Conditions for Antigen Fixation and Antigen-antibody Reaction

Primary antibody	Fixation	Incubation status	Origin of antibody
EGFR 1	methanol/acetone 1:1, -20°C , 5 min	1:100 dilution, 4°C , overnight	mouse anti-EGFR monoclonal antibody, DAKO
EGFR 1005	methanol/acetone 1:1, -20°C , 5 min	1:100 dilution, room temperature, 1 h	rabbit anti-EGFR polyclonal antibody, Santa Cruz
CS 1-4 (anti-LMP-1)	methanol/acetone 1:1, -20°C , 3 min	1:50 dilution, 4°C , overnight	mouse anti-LMP-1, DAKO

mal nasopharyngeal epithelium (internal control, when available) and the tumor (semi-quantitatively). If normal nasopharyngeal mucosa was not present on the same specimen, the comparison was made between an oral cancer specimen (external control known to over-express EGFR) and the nasopharyngeal cancer. If both authors did not assign the same grading, another author (Dr. Ko) made the final decision.

Indirect dual immunofluorescence assay in NPC biopsies An indirect dual immunostaining assay was designed to study whether LMP-1 and EGFR were co-expressed in NPC tissues. Twenty frozen sectioned NPC tissues, immunopositive for both LMP-1 and EGFR, were fixed in methanol-acetone mixture (volume/volume=1:1) at -20°C for 3 min. Then, the test samples were incubated with anti-LMP-1 (CS 1-4)¹⁵⁾ monoclonal antibody at a dilution of 1:10 overnight at 4°C . Next, they were incubated with indocarbocyanine (Cy3)-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) at room temperature, at a dilution of 1:100, for 1 h. Then, the samples were left to react with rabbit anti-EGFR polyclonal antibody (EGFR 1005) at a dilution of 1:80, at room temperature, for 1 h. Finally, the test samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel Research Products, Durham, NC) at a dilution of 1:400, at room temperature, for 1 h. The test samples were thoroughly washed with TBS for 15 min at room temperature after each incubation with an antibody. The test sample slides were examined under a Nikon fluorescence microscope equipped with both B-2A and G-2A filters. Switching the filters enabled the examination of specimens under green or blue light. A red fluorescence under excitation with green light represented a positive staining of LMP-1, and a green fluorescence under excitation with blue light represented a positive staining of EGFR. Immunostaining of the same specimens, but omitting both primary antibodies, was used as the negative control.

RESULTS

Demonstration of LMP-1 expression in NPC tissues by immunohistochemistry In order to identify EBV-encoded LMP-1 expression in NPC tumors, immunohistochemical staining of LMP-1 was performed using the CS 1-4 monoclonal antibody by the AP method. A red color in the cell membrane and cytoplasm represented a positive reaction. LMP-1 was detected in the tumor part only; infiltrating lymphocytes and stroma were not stained (Fig. 1a). Generally, the tumor nests were stained, but with some degree of heterogeneity. These data indicated that LMP-1 was expressed by NPC tumor cells, but the amount varied from cell to cell. Of the 60 specimens examined, 41 cases (68.3%) showed positive staining.

Expression of EGFR in NPC tissues Immunohistochemical stainings of EGFR were performed using EGFR 1 mouse anti-EGFR monoclonal antibody and EGFR 1005 rabbit anti-EGFR polyclonal antibody, by the ABC method. Brown color in the cell membrane or cytoplasm represented a positive reaction. In the skin and normal respiratory mucosa, the basal and parabasal layers demonstrate faint staining of EGFR. In this study, "over-expression of EGFR" was defined when the immunostaining of EGFR showed a similar intensity to that of the oral cancer specimen. In the 60 NPC specimens examined, 44 cases (73.3%) showed marked over-expression of EGFR in the tumor part (Fig. 1, b and c). The EGFR, similar to LMP-1, was distributed in the tumor nests, whereas infiltrating lymphocytes and stroma were negative for EGFR. The immunostaining results for EGFR 1 and EGFR 1005 were quite consistent: however, another 10 specimens were excluded from this study because of inconsistent results in the immunostainings of EGFR 1 and EGFR 1005.

Correlation between LMP-1 and EGFR in NPC tissues Statistical significance of differences among categorical data was evaluated using the χ^2 test with Yate's correction. A significant correlation was found between the expression of LMP-1 and EGFR ($P < 0.001$, χ^2 test, d.f.=1) (Table II). Regarding the histopathological classification, 7 specimens were keratinizing carcinoma, 14 were differentiated non-keratinizing carcinoma, and 39 were undifferentiated non-keratinizing carcinoma. There was no evident correlation between the expression of LMP-1 and WHO histopathologic classification.³³⁾

Demonstration of co-expression of LMP-1 and EGFR in NPC tumors by indirect dual immunofluorescence assay The immunohistochemical and statistical examinations revealed a close association between the expressions of LMP-1 and EGFR. Previous *in-vitro* transfection assays have indicated that LMP-1 could upregulate EGFR. To elucidate further the association between LMP-1 and EGFR *in vivo* and *in situ*, we conducted an indirect dual immunofluorescence assay in 20 NPC specimens. After sequential dual staining, the specimens were examined under a Nikon fluoromicroscope equipped with B-2A and G-2A filters. LMP-1 and EGFR could be detected on the same specimen by changing the filters. A red fluorescence under excitation with green light represented positive staining of LMP-1; a green fluorescence under excitation with blue light represented positive staining of EGFR. LMP-1 staining showed a membranous distribution (Fig. 2, a and b), and co-occurred with the green fluorescence of EGFR staining (Fig. 2, c and d). At a higher magnification, the staining of LMP-1 exhibited a granular pattern mainly in the cell membrane (Fig. 3a); this was different from the homogeneous staining of EGFR on the cell membrane (Fig. 3b). The results of immunofluorescence were

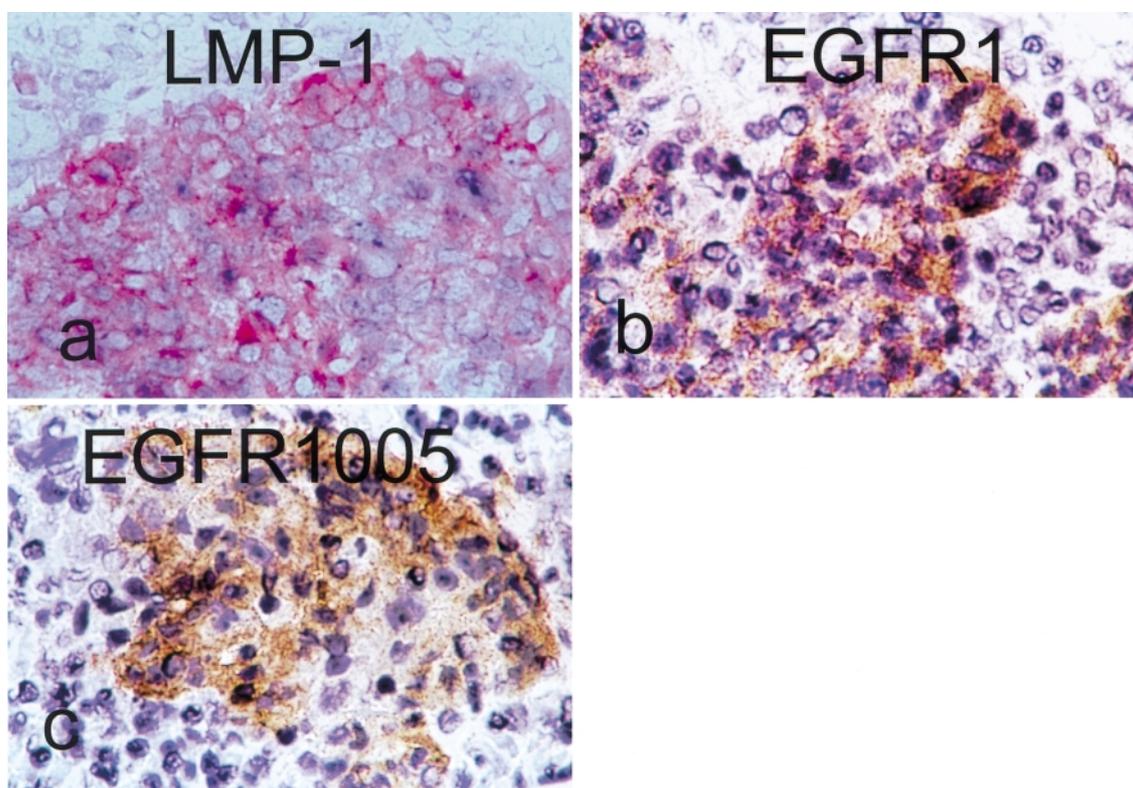


Fig. 1. Immunohistochemical assay for nasopharyngeal carcinoma. a: Staining of LMP-1 with CS 1-4 monoclonal antibody by the AP method, using new Fuchsin as the chromogen. The tumor nest was characterized by cells with irregular and large nuclei. The tumor nest was heavily stained, whereas the nearby stroma was not stained. b: Staining of EGFR with EGFR 1 monoclonal antibody by the ABC method, using DAB as the chromogen. As in the case of LMP-1 immunostaining, the tumor nest was heavily stained. c: Staining of EGFR with EGFR 1005 polyclonal antibody by the ABC method, using DAB as the chromogen. (original magnification $\times 200$)

Table II. Statistical Analysis of Immunostainings for LMP-1 and EGFR in 60 NPC Biopsies

	LMP-1 positive (cases)	LMP-1 negative (cases)	P value ^{a)}
EGFR over-expression			significant
positive	37	7	$P < 0.001$ ^{b)}
negative	4	12	
WHO classification (45)			non-significant
keratinizing	6	1	
non-keratinizing			
-differentiated	8	6	
-undifferentiated	27	12	

a) χ^2 test.

b) χ^2 test with Yate's correction.

consistent with those of immunohistochemistry, moreover, immunofluorescence disclosed a co-expression of LMP-1 and EGFR in NPC tumors.

In summary, the results of immunostaining revealed that, of 60 NPC specimens, 44 (73.3%) over-expressed EGFR; 41 (68.3%) were immunopositive for LMP-1. Morphologically, LMP-1 and EGFR were homogeneously present in the tumor nests. Statistically, the expression of EGFR was significantly related to that of LMP-1. Indirect dual immunofluorescence further demonstrated the co-expression of LMP-1 and EGFR in NPC. Our results thus establish the co-expression of EBV-encoded LMP-1 and EGFR, *in vivo* and *in situ*, in NPC tumors.

DISCUSSION

The role of EBV in the pathogenesis of NPC is still controversial. In B lymphocytes, EBV infection can induce immortalization of B-cells to LCLs. The LCLs express many of the EBV-encoded proteins, i.e., EBNA 1, EBNA 2, EBNA 3A, EBNA 3B, EBNA 3C, LMP-1, LMP-2A, LMP-2B and so on. In this type III latency of EBV infection,³⁴⁾ EBNA 2, EBNA 3A, EBNA 3C, LMP-1 are known to be essential in the process of B-cell

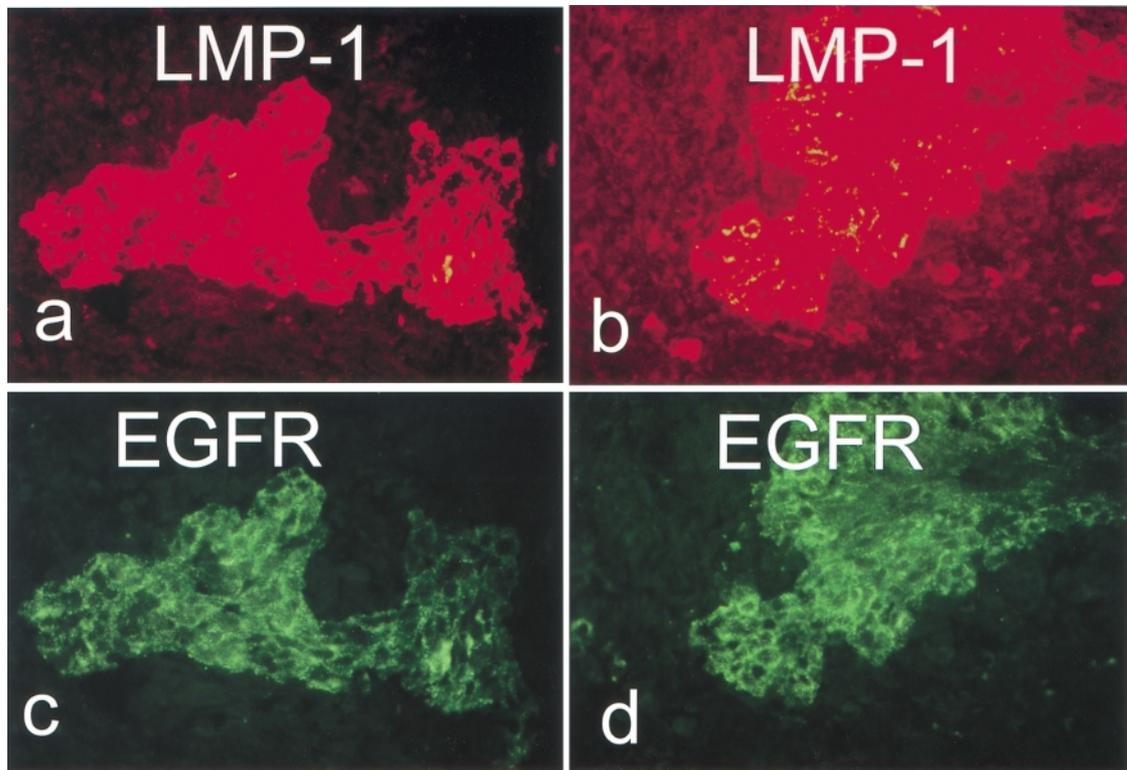


Fig. 2. Immunohistochemistry, morphologically and statistically, revealed a close association between EBV-encoded LMP-1 and EGFR in NPC. To assess further their association *in situ*, an indirect dual immunofluorescence assay was conducted. a, b: Staining of LMP-1 with CS 1-4 mouse anti-LMP-1 monoclonal antibody and “CY3”-conjugated goat anti-mouse IgG. c, d: Staining of EGFR with EGFR 1005 rabbit anti-EGFR polyclonal antibody and FITC-conjugated goat anti-rabbit IgG. (original magnification $\times 100$)

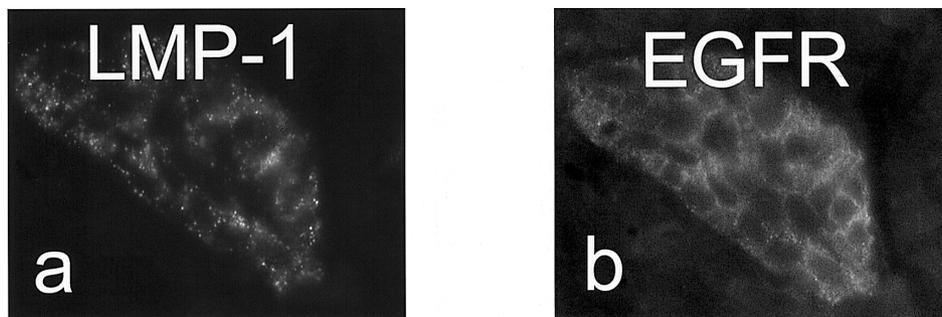


Fig. 3. Higher magnification of indirect dual immunofluorescence revealed the details of both stainings. a: Staining for LMP-1 exhibits a granular pattern on the cell membrane. b: Staining for EGFR displays a homogeneous membranous distribution different to that of LMP-1. (original magnification $\times 400$)

immortalization.^{8–10} In EBV-infected epithelial cells, such as NPC, the expression of EBV-encoded latent proteins is very restricted. Only EBNA 1, LMP-1, LMP-2A, LMP-2B are detected.^{34, 35} In this type II latency of EBV infection, LMP-1 is the most abundantly expressed transforming

protein. The expression of LMP-1 in NPC tumors ranged from 38 to 100% depending on the method and materials used.^{7, 11–14} By immunohistochemistry or immunoblotting, LMP-1 was detected at a lower rate than was obtained from reverse transcription polymerase chain reaction. In

this study, the immunopositive rate of LMP-1 in NPC was 68.3%, and the expression of LMP-1 showed a homogeneous distribution in the tumor nests.

Several reports have demonstrated the following effects of LMP-1 on human cells: LMP-1 could induce A20 zinc finger protein expression by activating NF- κ B in B lymphocytes²⁰; LMP-1 could transactivate p53 through the induction of NF- κ B in B cells²¹; and LMP-1 could upregulate EGFR in epithelial cells.^{31, 36, 37} How does LMP-1 influence these processes? Recently, some signal transduction pathways of LMP-1 have been characterized. In the cytoplasmic C-terminal of LMP-1, two "C-terminal activation domains (CTAR)" have been defined. The distal domain (CTAR-2) located between amino acid residues 351–385 is responsible for about 75% of the NF- κ B activity, and the proximal domain (CTAR-1) located between amino acid residues 194–232 is responsible for about 25% of the NF- κ B activity.^{37, 38} In addition, CTAR-1 also induces the expression of EGFR through TRAFs in epithelial cells.³⁷

EGF and EGFR are essential for the growth of epithelial cells. Aberrant expression of these molecules might contribute to the carcinogenesis or the progression of epithelial malignancies.²⁷ Several reports have revealed increased production of EGFR protein in both fresh tumors and cell lines derived from patients with squamous cell carcinoma of the head and neck.^{30, 39} The significance of this over-production is unclear, though in a number of tumor systems, increased EGFR mRNA has been associated with an advanced tumor stage or a worse prognosis.⁴⁰ The expression of EGFR in NPC has not been widely examined. Zheng *et al.* first noted the strong immunostaining of EGFR in 14 out of 23 (60.8%) NPC biopsies.¹⁴ They also noted that there seemed to be a correlation between the expressions of EGFR and LMP-1. Roychowdhury *et al.* found that EGFR was expressed in

73.1% of NPC tumors.⁴¹ In this study, over-expression of EGFR was observed in 73.3% of NPC tumors. All of these results suggest that over-expression of EGFR in NPC is a common phenomenon, and might contribute to the carcinogenesis of NPC.

In-vitro studies have shown that the tumorigenic effect of LMP-1 on epithelial cells is probably mediated through the upregulation of EGFR. Whether this effect exists and plays some role in the tumorigenesis of NPC has not been documented. In this study, immunohistochemistry showed that both LMP-1 and EGFR were homogeneously expressed in NPC tumor nests. A significant association was also shown by statistical analysis. Moreover, the immunofluorescence findings demonstrated that LMP-1 and EGFR were co-expressed in NPC tumors. Thus, we have established the co-expression of LMP-1 and EGFR, *in vivo* and *in situ*, in NPC. These data support the hypothesis that EBV plays some role in the tumorigenesis of NPC through the effects of LMP-1 and EGFR. Eleven NPC specimens were immunonegative for either LMP-1 or EGFR, so other tumorigenic mechanisms might be involved in those cases, or the sensitivity of immunostainings may have been insufficient. Confirmation of the correlation between LMP-1 and EGFR by means of more sensitive methods would be desirable.

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