J Microbiol Immunol Infect
2002;35:136-140

Expression of Epstein-Barr virus latent membrane protein 1 and B-cell leukemia-lymphoma 2 gene in nasopharyngeal carcinoma tissues

John Jenn-Yenn Lu[1,2] Chi-Long Chen[3,5], Tsuey-Ying Hsu[2], Jen-Yang Chen[2,4], Ih-Jen Su[3], Winston CY Yu[4], Czau-Siung Yang[2]

[1]National Taichung Nursing College, Taichung, and School of Medical Technology, Taipei Medical University, Taipei; Graduate Institutes of [2]Microbiology and [3]Pathology, College of Medicine, National Taiwan University; [4]National Health Research Institute, Taipei; and [5] Department of Pathology, China Medical College Hospital, Taichung, Taiwan, ROC

Received: September 18, 2001 Revised: November 23, 2001 Accepted: February 19, 2002

Co-expression of B-cell leukemia-lymphoma 2 gene (Bcl-2) and Epstein-Barr virus latent membrane protein 1 (LMP-1) in nasopharyngeal carcinoma tissues were tested using immunohistochemical methods. Results showed that there were 32% (14/44) and 68% (30/44) LMP-1 and Bcl-2-positive cases, respectively. Among the LMP-1-positive tissues, 8 (57%) of 14 specimens were also Bcl-2-positive. The level of LMP-1 and Bcl-2 expression was associated with the clinical stages of nasopharyngeal carcinoma. Furthermore, when LMP-1- and Bcl-2-positive cases were combined, the highest positive score was found in clinical stage II as well as in the early stage (stages I and II) of nasopharyngeal carcinoma. While further studies with more cases are needed, this study suggests that co-expression of Bcl-2 and LMP-1 may be involved in the process of nasopharyngeal carcinoma aggravation.

Key words: B-cell leukemia-lymphoma 2 gene (Bcl-2), Epstein-Barr virus, latent membrane protein 1 (LMP-1), nasopharyngeal carcinoma

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis [1].The virus is also associated with monoclonal B lymphoproliferative disease [2], endemic Burkitt's lymphoma (BL) [3], Hodgkin's disease [4], unusual T-cell lymphoma [5], nasopharyngeal carcinoma (NPC) [6,7] and gastric carcinoma [8].

Nasopharyngeal carcinoma is one of the most common malignant head and neck tumor affecting people in southern China, including those in the Taiwan area, and emigrants from that region. Environmental [9] and genetic [10] factors have been studied and are found to be associated with NPC. The results obtained from epidemiologic, immunologic, and virologic studies have established a close relationship between EBV and NPC.

Latent membrane protein 1 (LMP-1), one of the latent gene products of EBV, is a 62-kD integral membrane protein [11]. Latent membrane protein-1 forms discrete patches in the cytoplasm of latently EBV-infected B lymphocytes [11] and associates with the cytoskeletal intermediate filament protein, vimentin [12]. Latent membrane protein-1 is essential for B-cell growth transformation [13]. Introduction of the LMP-1 gene into the cells increases expression of the *bcl-2* proto-oncogene [14], thereby preventing the apoptosis of EBV-negative sporadic BL cells. Latent membrane protein-1 also acts as an oncogene in rodent fibroblasts, in which it can transform NIH 3T3 cells, leading to the loss of anchorage dependence and lesser serum requirement, and ultimately rendering the cells tumorigenic in nude mice [15].

The B-cell leukemia and lymphoma 2 gene (Bcl-2) is located on chromosome 18q21, but the t(14;18) translocation juxtaposes the gene to the immunoglobulin heavy chain loci on chromosome 14 [16]. Bcl-2 is expressed in all precursors of the hematopoietic lineage and functions as a cell death suppressor [16].

In a previous study, we demonstrated that over-expression of LMP-1 induced apoptosis of epithelial cells [17], but did not affect the expression of *bcl-2* [17]. This situation is different from that seen in LMP-1 transfected B-cell line, where LMP-1 upregulates the expression of *bcl-2* and protects B cells from apoptosis [14]. A recent study showed that LMP-1 cooperates with *bcl-2*, resulting in transformation of human epithelial

Corresponding author: Dr. Czau-Siung Yang, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan, ROC. Current address of Dr. John Jenn-Yenn Lu: Department of Basic Medical Science, National Taichung Nursing College, 193, Section 1, San-Min Road, Taichung, Taiwan, ROC. E-mail: johnlu@ntcnc.edu.tw

cells *in vitro* [18]. The expression of LMP-1 was detected in 30% to 65% of NPC tissues [19], and 80% of NPC biopsies was reported to be *bcl-2*-positive [20]. We hypothesized that co-expression of Bcl-2 and LMP-1 may have an important role in the oncogenic process of NPC. To test this hypothesis, immunohistochemistry assay was performed to detect the expression of Bcl-2 and LMP-1 in NPC biopsies.

Materials and Methods

Forty-four EBV-positive NPC samples were obtained from the Department of Pathology, National Taiwan University Hospital. The EBV genome was detected by *in situ* hybridization method [21]. The clinical staging of NPC was based on the TNM staging method as indicated in the Manual of American Joint Committee on Cancer [22].

In this study, the samples were formalin-fixed and paraffin-embedded. Immunostaining of NPC tissues was performed using a peroxidase-labeled streptavidin-biotin detection system (LSAB, Dako, Carpinteria, CA, US) [23] with antibodies to LMP-1 (CS1-4, Dako, Denmark) and Bcl-2 (clone 124, Dako, Denmark). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and the sections were pretreated by boiling for 10 min. The antigenicity was enhanced and false nuclear reactivity was diminished by pre-treatment with 1 mg/mL of pronase E in phosphate-buffered saline for 5 min at 37°C [24]. The primary antibodies, CS1-4 (1:100 dilution) and anti-Bcl-2 (1:50 dilution), were applied overnight at 4°C followed by incubation with secondary biotinylated rabbit anti-mouse immunoglobulin G antibody (Dako, Denmark) for 20 min. A streptavidin-biotin-alkaline phosphatase complex reagent and streptavidin-biotin-peroxidase complex reagent for LMP-1 and Bcl-2, respectively, were then added for 20 min at room temperature. The sections were thoroughly washed with phosphate-buffered saline between the steps. New fuchsin and diaminobenzidine tetrahydrochloride were used as the chromogen for LMP-1 and Bcl-2, respectively. The sections were then countered with hematoxylin, mounted and observed under a microscope. Appropriate positive and negative controls were also included.

Results and Discussion

Immunohistochemically positive staining of LMP-1 in the tissue appeared as intracytoplasmic red staining

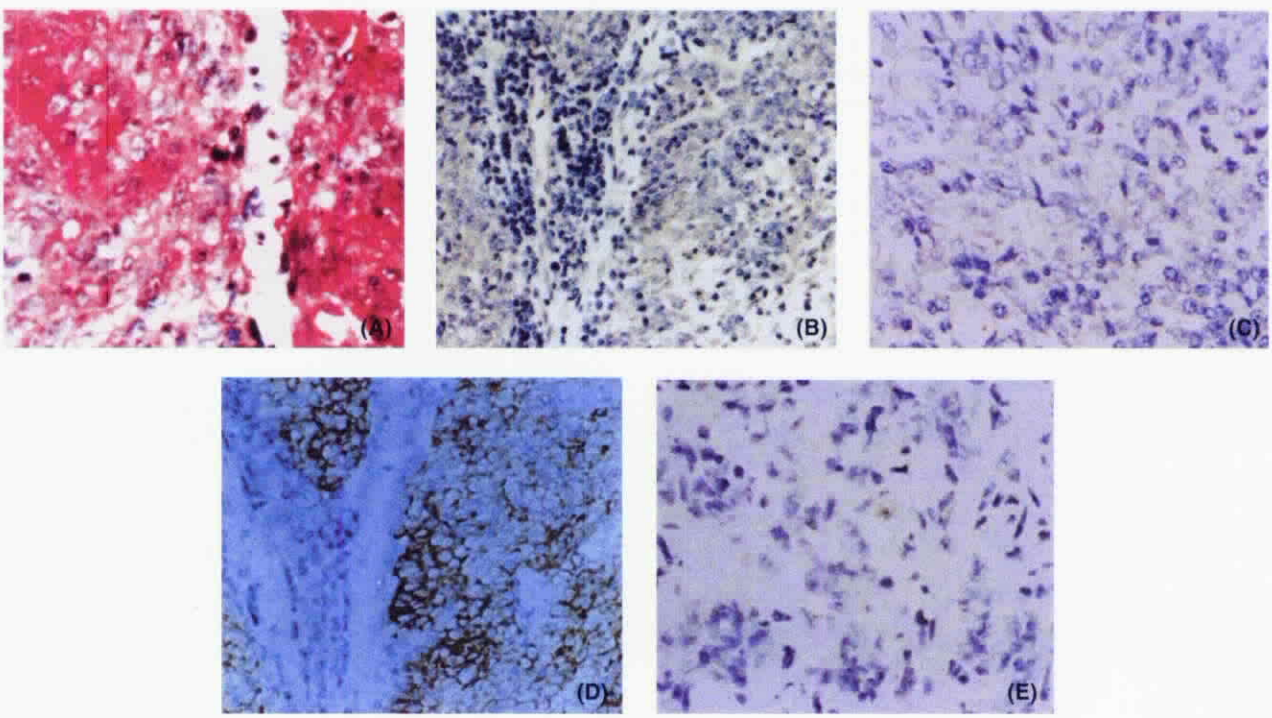

Fig. 1. Immunocytochemical analysis of paraffin-embedded NPC tissues. **(A)** Latent membrane protein 1-positive tissue. **(B)** Latent membrane protein 1-negative tissue. **(C)** Latent membrane protein 1 and B-cell leukemia-lymphoma 2 gene double-negative control. **(D)** B-cell leukemia-lymphoma 2 gene-positive tissue. **(E)** B-cell leukemia-lymphoma 2-negative tissue.

Table 1. Epstein-Barr virus latent membrane protein 1 and B-cell leukemia-lymphoma 2 gene expression in different clinical stage of nasopharyngeal carcinoma

Patient no.	Sex	EBV-DNA	LMP-1	Bcl-2	Stages of NPC[a]
1	F	++[b]	–	+	IV
2	M	++	–	–	I
3	F	+++	–	+	II
4	M	+	+++	+	II
5	M	++	++	++	I
6	M	+	–	–	III
7	F	+++	+++	–	II
8	M	+	+++	+	II
9	M	++	–	–	III
10	M	+	+	+	II
11	M	+	++	+	II
12	M	++	–	+	II
13	M	+	–	–	III
14	M	++	–	+	II
15	M	+	–	+	II
16	F	++	+	–	II
17	M	++	+	+	II
18	F	+	–	–	III
19	M	++	++	++	I
20	M	+	+	+	IV
21	M	+	–	++	I
22	M	+	–	++	IV
23	M	++	–	+++	I
24	M	++	–	–	III

Abbreviations: EBV = Epstein-Barr virus; LMP = latent membrane protein; Bcl-2 = B-cell leukemia-lymphoma 2 gene; NPC = nasopharyngeal carcinoma; M = male, F = female

[a]Based on the American Joint Committee on Cancer staging manual method.

[b]+++: strong reactivity; ++: moderate reactivity; +: weak reactivity; –: not detected.

throughout the tumor cells (Fig. 1, panel A), and negative staining of LMP-1 did not show any signal in the tissue (Fig. 1, panel B). Latent membrane protein 1 and Bcl-2 negative tissues were used as negative control (Fig. 1, panel C). Positive staining of Bcl-2 in paraffin embedded tissue appeared as cytoplasmic brownish color throughout the tumour cells (Fig. 1, panel D), whereas negative staining of Bcl-2 did not show any brownish signal (Fig. 1, panel E). Immunohistochemical results revealed that the expression of Bcl-2 was found in 30 (68%) of 44 NPC samples. In some positive cases, the immunoreactivity of Bcl-2 was heterogeneous within the tumour area; some were strongly stained, and others were weakly stained. The LMP-1 positive tissues were detected in 14 (32%) of 44 NPC samples. Among 14 LMP-1-positive NPC tissues, 8 (57%)

Table 2. Expression of latent membrane protein 1/B-cell leukemia-lymphoma 2 gene in different clinical stages of nasopharyngeal carcinoma

	Stage I	Stage II	Stage III	Stage IV
LMP-1 (+)	6[a]	21	0	2
(–)	3[a]	4	5	2
Bcl-2 (+)	13[a]	18	0	7
(–)	1[a]	2	5	0
LMP-1 (+) / Bc1-2 (+)	19[a]	39	0	9
LMP-1 (–) / Bc1-2 (–)	4[a]	6	10	2

Abbreviations: LMP = latent membrane protein; Bcl-2 = B-cell leukemia-lymphoma 2 gene; NPC = nasopharyngeal carcinoma

[a]Sum of scores of LMP-1 or Bcl-2 expression.

Table 3. Association of latent membrane protein 1/B-cell leukemia-lymphoma 2 gene expression with the clinical stages of nasopharyngeal carcinoma

	Clinical stages[a]		Sum of scores	Odds Ratio	*p* value
	Early	Late			
LMP-1 (+)	27[b]	2[b]	29	13.5	0.0049[e]
(–)	7[b]	7[b]	14		
Bcl-2 (+)	31[c]	7[c]	38	7.38	0.020[e]
(–)	3[c]	5[c]	8		
LMP-1 (+) / Bcl-2 (+)	58[d]	9[d]	67	7.73	0.0003[f]
LMP-1 (–) / Bcl-2 (–)	10[d]	12[d]	22		

Abbreviations: LPM = latent membrane protein; Bcl-2 = B-cell leukemia-lymphoma 2 gene; NPC = nasopharyngeal carcinoma
[a]Early: stages I and II; Late: stages III and IV.
[b] Sum of scores of LMP-1 expression.
[c]Sum of scores of Bcl-2 expression.
[d]Sum of scores of LMP-1 and Bcl-2 expression.
[e]Fisher's exact test.
[f] χ^2 test with Yate's correction.

samples were also Bcl-2 positive (Table 1). Because 4 of 14 LMP-1 positive cases had no clinical staging record, they were not included in the Table.

To further examine the association of the expression of LMP-1 and Bcl-2 with the clinical stages of NPC, we compared the clinical stages of NPC with the intensities of LMP-1 and Bcl-2 expression. As shown in Table 1, the level of Bcl-2 expression was not associated with that of LMP-1 expression, and this result was similar to our previous finding that LMP-1 did not up-regulate Bcl-2 expression *in vitro* [18]. In addition, an earlier study showed that the ectopic expression of Bcl-2 efficiently inhibited the LMP-1-induced apoptotic effect in epithelial cells, and that Bcl-2 cooperated with LMP-1 to induce cellular transformation [19]. These findings suggest that the cooperation of Bcl-2 and LMP-1 may have an important role in the processes of EBV-associated tumorigenesis of epithelial origin. For comparison, the stages I and II of NPC were combined to represent an early clinical stage of NPC, and the stages III and IV were combined as a late clinical stage. In addition, the levels of LMP-1 or Bcl-2 staining were scored as follows: (–) as 1, (+) as 2, (++) as 3, and so on. The total numbers of LMP-1 and Bcl-2 scores were summed up. Table 2 summarizes the summed scores of LMP-1 and Bcl-2 corresponding to the clinical staging of NPC. The association of LMP-1 and Bcl-2 expression with the clinical stages of NPC was analyzed by Pearson's chi-square test. The results were shown in Table 4, in which a higher level of LMP-1 expression was observed in the early clinical stage of NPC (odds ratio, 13.5; p=0.0026). The intensity of Bcl-2 staining is also associated with the early clinical stage of NPC (odds ratio, 7.38; p=0.02) (Table 3). When LMP-1- and Bcl-2-positive scores were combined, the highest score was found in clinical stage II (Table 2) as well as in the early stage (stages I and II) of NPC (odds ratio, 7.73, p=0.0003) (Table 3). The results suggest that co-expression of LMP-1 and Bcl-2 may be involved in the process of NPC aggravation.

Acknowledgments

We sincerely thank Dr. Jenq-Yuh Ko and Dr. Der-Huei Yeh (Department of Otolaryngology, National Taiwan University Hospital) for their generous help in diagnosing and staging the NPC cases in this study.

References

1. Henle G, Henle W, Diehl V. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc Natl Acad Sci USA 1968;59:94-101.
2. Young, L, Alfieri C, Hennessy K, Evans H, O'Hara C, Anderson KC, Ritz J, Shapiro RS, Rickinson AB, Kieff E, Cohen JI. Expression of Epstein-Barr virus transformatiom-associated genes in tissues of patients with EBV lymphoproliferative disease. N Engl J Med 1989;321:1080-5.
3. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet 1964;1:702-3.
4. Herbst H, Dallenbach F, Hummel M, Niedobitek G, Pileri S, Muller-Lantzsch N, Stein H. Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. Proc Natl Acad Sci USA 1991;88:4766-70.
5. Jones JF, Shurin S, Abramowsky C, Tubbs RR, Sciotto CG, Wahl R, Sands J, Gottman D, Katz BZ, Sklar J. T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. N Engl J Med 1988;318: 733-41.
6. Old LJ, Boyse WA, Oettgen HF, Deharven E, Geering G, Williamson B, Clifford P. Precipitating antibodies in human serum to an antigen present in cultured Burkitt's lymphoma cells. Proc Natl Acad Sci USA 1966;56:1699-704.
7. Zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Stantesson L. Epstein-Barr virus DNA in biopsies of Burkitt tumors and anaplastic carcinoma of nasopharynx.

Nature 1970;228:1056-8.
8. Shibata D, Tokunaga M, Uemura Y, Sato E, Tanaka S, Weiss LM. Association of Epstein-Barr virus with undifferentiated gastric carcinomas with intense lymphoid infiltration lymphoepithelioma-like carcinoma. Am J Pathol 1991;139: 469-674.
9. Yu MC, Ho JHC, Lai SH, Henderson BE. Cantonese-style salted fish as a cause of nasopharyngeal carcinoma: report of a case-control study in Hong Kong. Cancer Res 1986;46:956-61.
10. Chan SH, Day NE, Kunaratnam N, Chia KB, Simons MJ. HLA and nasopharyngeal carcinoma in Chinese: a further study. Int J Cancer 1983;32:171-6.
11. Liebowitz D, Wang D, Kieff E. Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. J Virol 1986;58:233-7.
12. Liebowitz D, Kopan R, Fuchs E, Sample J, Kieff E. An Epstein-Barr virus transforming protein associates with vimentin in lymphocytes. Mol Cell Biol 1987;7:2299-308.
13. Kaye KM, Izumi KM, Kieff E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci USA 1993;90:9150-4.
14. Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A. Induction of *bcl-2* expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 1991;65:1107-15.
15. Baichwal VR, Sugden B. Transformation of Balb/3T3 cells by the *BNLF-1* gene of Epstein-Barr virus. Oncogene 1988;2:461-7.
16. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM. The t (14;18) chromosome translocation involved in B-cell neoplasms result from mistakes in VDJ joining. Science 1985;229:1390-3.
17. Lu JJY, Chen JY, Hsu TY, Yu WCY, Su IJ, Yang CS. Induction of apoptosis in epithelial cells by Epstein-Barr virus latent membrane protein 1. J Gen Virol 1996;77:1883-92.
18. Lu JJY, Chen JY, Hsu TY, Yu WCY, Su IJ, Yang CS. Cooperative interaction between Bcl-2 and Epstein-Barr virus latent membrane protein 1 in the growth transformation of human epithelial cells. J Gen Virol 1997;78:2975-85.
19. Fahraeus R, Fu LF, Ernberg I, Finke I, Rowe M, Klein G, Falk K, Nilsson E, Yadav M, Busson P, Tursz T, Kallin B. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 1988;42:329-38.
20. Lu QL, Elia G, Lucas S, Alero Thomas J. Bcl-2 proto-oncogene expression in Epstein-Barr virus-associated nasopharyngeal carcinoma. Int J Cancer 1993;53:29-35.
21. Chen CL, Wen WN, Chen JY, Hsu MM, Hsu HC. Detection of Epstein-Barr virus genome in nasopharyngeal carcinoma by *in situ* DNA hybridization. Intervirology 1993;36:91-8.
22. Fleming ID, Cooper JS, Henson DE, Hutter RVP, Kennedy BJ, Murphy GP, O'sullivan B, Sobin LH, Yarbro JW, eds. AJCC Cancer Staging Manual. 5th ed. Lippincott-Raven; 1997:32-5.
23. Elias JM, Margiotta M, Gaborc D. Sensitivity and detection efficiency of peroxidase antiperoxidase (PAP), avidin-biotin peroxidase complex (ABC) and peroxidase-labeled avidin-biotin (LAB) methods. Am J Clin Pathol 1989;92:62-7.
24. Gulley ML, Pulitzer DR, Eagan PA, Schneider BG. Epstein-Barr virus infection is an early event in gastric carcinogenesis and is independent of *bcl-2* expression and p53 accumulation. Hum Pathol 1996;27:20-7.