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Papers

Differences in the Growth Pattern and Clinical Course of EBV-LMP1 Expressing and Non-expressing Nasopharyngeal Carcinomas

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All low differentiated or anaplastic forms of nasopharyngeal carcinoma (NPC) carry multiple copies of EBV-DNA and express EBNA1. The major membrane protein, LMP1, is only expressed in 65% of the tumours. The physiological function of LMP1 in the viral life cycle is unknown, but it has been shown to transform established rodent fibroblasts and immortalised human keratinocytes *in vitro*, and to increase the likelihood of a malignant transformation. We studied 74 cases collected from the Shanghai and Guanzhou areas in China. LMP1 expression was assessed in tumour biopsies by immunoblotting. Clinical and follow-up data were evaluated according to the classification of WHO. The laboratory and the clinical data were assembled in a mutually independent double blind fashion. Our findings indicate that the LMP1-positive tumours grew faster and more expansively than LMP1-negative tumours, but nevertheless had a better prognosis. LMP1-negative tumours recurred at a higher frequency, and showed an increased tendency to metastasise.

Key words: EBV-LMP1, clinical, NPC
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INTRODUCTION

VIRTUALLY ALL low differentiated or anaplastic nasopharyngeal carcinomas (NPC) carry the EBV genome, in multiple copies [1, 2]. NPC cells regularly express EBNA1, do not express the immunoblast-associated EBNA2-6, but express the main virally encoded membrane protein, LMP1, in 65% of cases [3, 4]. The remaining 35% are LMP1-negative. LMP1 negativity is due to down-regulation of LMP1 expression, associated with methylation of the regulatory (LRS) 5′ flanking region [5].

The function of the LMP1 protein in the interaction between virus and host is unknown. Its regular and relatively abundant expression in EBV transformed B-cells suggests that it has important and probably multiple functions. In experimental model systems, LMP1 has been shown to transform immortalised rodent fibroblasts [6, 7] and human keratinocytes *in vitro* [8] and to induce certain phenotypic changes in EBV-negative B-cell lines [9, 10]. Moreover, it has been shown that LMP1 performs an essential function in the viral immortalisation of B-cells [11].

In view of the involvement of LMP1 in epithelial cell transformation [8] and its demonstrated immunogenicity both in human and animal model systems [12, 13], we have compared a number of clinical features between LMP1 expressing and non-expressing NPC cases, collected in the high endemic Guangdong and Shanghai areas of China. A total of 74 patients were followed over observation periods between 2.5 and 3 years. Our findings indicate that LMP1-positive tumours may grow faster and more expansively than their LMP1-negative counterparts, but that, nevertheless, they have a better prognosis.

MATERIALS AND METHODS

Tumours

NPC tumour biopsies, collected from 74 different patients, have been obtained from The Shanghai Cancer Hospital, The ENT Hospital and The Guanzhou Provincial Hospital, prior to treatment. Their LMP1 expression was examined in Sweden. Diagnosis was based on a histopathological examination, performed by the pathologist at each hospital according to the WHO classification [14]. Biopsy specimens were snap-frozen within 1 h of surgical removal and stored at −70°C.

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Classification of NPC

All tumours were undifferentiated carcinomas. The tumour cells showed no evidence of squamous differentiation, and were usually strongly infiltrated with lymphocytes.

Clinical staging of NPC

The clinical staging of NPC was performed according to TNM classification. The extension of the primary tumour was indicated as tumour stage, T. For T0, the primary tumour was invisible, but histological examination showed a precancerous state. T1 indicated that the tumour was confined to one wall of the nasopharynx. In T2, the tumour had extended beyond two walls, but remained confined to the nasopharynx. In T3, tumour had extended beyond the nasopharynx or had involved the base of the skull. T4 designation was used when the two alternatives of T3 occurred together.

The N-series of designations indicates the involvement of the regional lymph nodes. N0 indicates cases without palpable lymph nodes. N1 designates the involvement of a single, homolateral lymph node that remains smaller than 2 × 2 cm. N2 indicates contralateral, homolateral or bilateral lymph nodes larger than 2 × 2 cm, but smaller than 8 × 8 cm, while N3 indicates lymph nodes larger than 8 × 8 cm or extending into supraclavicular area. All patients have been followed for at least 2.5 years. Recurrence was registered if any lymph node or distant metastasis was noted within 2.5 years after therapy.

LMP1 expression

Detection of LMP1 protein in the biopsies was by immunoblotting, as previously described [15]. Briefly, frozen tissues were homogenised in a small volume of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP-40, 0.1% sodium dodecyl sulphate, 1 mM phenyl methylsulphonylfluoride, PMSF) and sonicated (3 × 15 s) on ice. Extracts were heated at 90°C for 5 min, and clarified by centrifugation for 20 min at 10000 *g*. Aliquots of the supernatant were used for SDS–PAGE on 7.5% acrylamide gels in Mini-gel (Bio-Lab) at 15 V/cm.

Electrophoretic transfer to nitro-cellulose filter (Hybond N, Amersham) was at 10 V/cm, 45 min. The filters were stained with Ponceau S (Sigma), and the positions of the molecular weight markers (Bio-Rad) were noted. The amount of protein in each tumour was estimated by comparison with B95-8 or LCL samples. After destaining with water, the filters were pre-absorbed in phosphate-buffered saline containing 5% non-fat dry milk, and then incubated with antibody (S12, 1:1000 dilution and PG 1:10 dilution) for 2 h at room temperature or overnight at 4°C. Specifically bound IgG was enzymatically detected after binding of alkaline phosphatase conjugated antibody (Sigma).

All LMP1 negative tumours were tested for EBNA1 expression by immunoblotting with the polyclonal, polyvalent PG antibody as described previously [3].

The investigation was performed as a double blind test: the clinical investigators were not informed of the LMP1 tests, nor did the investigator carrying out the LMP1 test in Stockholm know of the clinical course at the time of the test. Codes were broken and results evaluated at the end of the study.

RESULTS

Clinical follow-up data were available for all 74 NPC biopsy donors. Patients with LMP1-positive and -negative biopsies were compared with regard to age, sex, clinical stage, size and site of tumour, lymph node involvement, metastasis and recurrence.

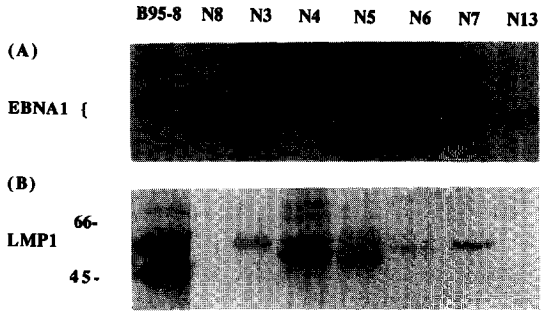

Figure 1. Examples of immunoblotting on tumour material from patients. In panel A, all NPC tumours regularly express EBNA1 detected by PG antibody. Panel B shows the immunoblotting with the S12 antibody on tumours from the same patients with LMP1-expressing and non-expressing biopsies. Size of LMP1 was estimated by standardised molecular weight.

Figure 1 illustrates the immunoblotting reaction of LMP1 and EBNA1. The majority of samples present a single band, corresponding to 63–66 kDa. Some NPC biopsy samples expressed two or three bands, probably due to partial degradation of LMP1. The smaller MW-size band seen in the B95-8 control in Figure 1B is due to the shorter lytic transcript in virus producer B95-8 cells, not found in NPC. The LMP1-negative tumours were tested at least twice, and those samples regularly expressed EBNA1.

Forty-nine of the 74 tumours were LMP1-positive and 25 were negative. This result (66% LMP1-positive) is in agreement with our earlier finding [3]. No statistically significant difference was found in age, sex and tumour type between patients with LMP1-positive and LMP1-negative tumours (data not shown). All three patients under 30 years of age were in the LMP1-positive group.

Table 1 summarises the results. All 74 cases were evaluated with regard to tumour stage and lymph node involvement, but only 48 of these were available for the evaluation of recurrence, requiring a minimum observation time of 2.5 years.

Table 1. Clinical parameters in patients with EBV-LMP1-expressing and non-expressing nasopharyngeal carcinomas

	Status	No.	LMP1+	LMP1−	χ square test *P* value (chi square value)
Tumour stage	T0	1	0	1	
	T1	1	0	1	
	T2	**40**	**23**	**17**	***P* < 0.025 (5.42)**
	T3	**22**	**19**	**3**	
	T4	10	7	3	
	T0–2	**42**	**23**	**19**	***P* < 0.025 (5.69)**
	T3–4	**32**	**26**	**6**	
Lymph node involvement	N0	31	13	18	
	N1	6	4	2	
	N2	26	22	4	
	N3	11	10	1	
	N0	**31**	**13**	**18**	***P* < 0.025 (14.00)**
	N1–3	**43**	**36**	**7**	
Recurrence	–	**30**	**25**	**5**	***P* < 0.025 (10.00)**
	+	**18**	**7**	**11**	

With regard to tumour stage, a significant difference was found between the T2 and T3 groups, with there being more LMP1-negative tumours in the less advanced T2 group than in the more advanced T3 stage. The difference was even more striking when the less advanced T0–2 stages were compared with the more advanced T3–4 stages. Two-sided values were used for statistical analysis. The difference was significant at the 0.025 level. It indicates that LMP1-positive tumours grow faster and more expansively than LMP1-negative tumours. The LMP1-positive tumours also tended to invade more frequently outside the nasopharynx than LMP1-negative tumours.

There were significant differences for lymph node involvement, with there being more LMP1-negative tumours in the N0 group, with no lymph node involvement, than in the N1–3 group. The difference was significant at the $P = 0.025$ level. These findings indicate that LMP1-positive tumours are more progressive and more prone to invade the lymph nodes than LMP1-negative.

In the group of 30 patients who showed no recurrence during the minimum observation period of 2.5 years, there were more LMP1-positive tumours whereas the opposite was found in the group of 18 patients whose tumours recurred within a similar period. The difference was significant at the $P = 0.025$ level. These findings indicate that LMP1-positive tumours have a lower tendency to recur.

DISCUSSION

Our findings show, surprisingly, that LMP1-positive NPCs tend to expand and progress more rapidly than their LMP1-negative counterparts, but are nevertheless less prone to recur. These seemingly paradoxical observations may be related to two, experimentally demonstrated, facts. Firstly, LMP1 can transform immortalised strains of rodent fibroblasts and human keratinocytes. If these experimental findings are relevant for nasopharyngeal carcinoma *in vivo*, they might explain the more expansive growth of LMP1-positive tumours *in vivo*. Secondly, LMP1 is immunogenic and *in vitro* can serve as the target of HLA class I restricted cytotoxic T lymphocytes generated by stimulation with autologous EBV transformed B-cells [13, 16], and can convert non-immunogenic mouse mammary adenocarcinoma cells to immunogenicity *in vivo* [12]. Since the lack of LMP1 expression in one-third of NPCs investigated was found to be associated with the methylation of the 5′flanking LRS region [5]. LMP1 negativity may represent a secondary change, representing a regulatory response of the tumour to immunoselection. By the time this response occurs during tumour progression, the NPC cells may have become independent of the proliferation-driving effect of LMP1 by secondary cellular changes. Our findings indicate that the loss of LMP1 expression may, nevertheless, reduce, to some extent, the expansiveness of the tumour *in vivo* while also reducing its immunogenicity.

Our findings are consistent with the fact that EBV-positive Burkitt lymphomas are more curable by chemotherapy and less generalising than EBV-negatives [17]. Another analogy can be found in the better prognosis of HPV-positive anogenital carcinoma, compared with their HPV-negative counterparts [18–20].

1. Klein G, Giovanella BC, Lindahl T, Fialkow PJ, Singh S, Stehlin JS. Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx. *Proc Natl Acad Sci USA* 1974, **71**, 4737–4741.
2. Andersson-Anvret M, Klein G, Forsby N, Henle W. The association between undifferentiated nasopharyngeal carcinoma and Epstein-Barr virus shown by correlated nucleic acid hybridization and histopathological studies. In De-The G, Ito Y, eds. *Nasopharyngeal Carcinoma: Etiology and Control*. Lyon, IARC, 1978, 347–357.
3. Fåhraeus R, Hu Li-Fu, Kallin B, *et al.* Expression of Epstein-Barr virus encoded proteins in nasopharyngeal carcinoma. *Int J Cancer* 1988, **42**, 329–338.
4. Young LS, Dowson CW, Rickinson AB, *et al.* Epstein-Barr virus gene expression in nasopharyngeal carcinoma. *J Gen Virol* 1988, **69**, 1051–1065.
5. Hu Li-Fu, Minarovits J, Cao S-L, Klein G, Ernberg I. Variable expression of latent membrane protein in nasopharyngeal carcinoma can be related to methylation status of the Epstein-Barr virus BNLF-1 5″-flanking region. *J Virol* 1991b, **65**, 1558–1567.
6. Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* 1985, **43**, 831–840.
7. Baichwal VR, Sugden B. Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. *Oncogene* 1988, **2**, 461–467.
8. Hu Li-Fu, Chen Fu, Zheng Xi, Ernberg I, Klein G, Winberg G. Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP1. *Oncogene* 1993, **8**, 1575–1583.
9. Wang D, Liebowitz D, Kieff E, *et al.* Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J Virol* 1988, **62**, 4173–4184.
10. Henderson S, Rowe M, Rickinson A, *et al.* Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* 1991, **65**, 1107–1115.
11. 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**, 9150–9154.
12. Trivedi P, Masucci MG, Winberg G, Klein G. The EBV encoded membrane protein LMP but not the nuclear antigen EBNA-1 induces rejection of the transfected murine mammary carcinoma cells. *Int J Cancer* 1991, **48**, 794–800.
13. Cuomo L, Trivedi P, Wang F, Winberg G, Klein G, Masucci MG. Expression of the Epstein-Barr virus encoded membrane antigen (LMP) increases the stimulatory capacity of EBV-negative B lymphoma lines in allogeneic mixed lymphocyte cultures. *Eur J Immunol* 1990, **20**, 2293–2299.
14. Shanmugaratnam K, Chan SH, De-The G, Gob JEH, Khor TH, Simons MJ. Histopathology of nasopharyngeal carcinoma. *Cancer* 1979, **44**, 1029–1044.
15. Kallin B, Dillner J, Klein G, *et al.* Four virally determined nuclear antigens are expressed in Epstein-Barr virus-transformed cells. *Proc Natl Acad Sci USA* 1986, **83**, 1499–1503.
16. Khanna R, Borrows S, Moss D, *et al.* Localisation of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J Exp Med* 1992, **176**, 169–176.
17. Magrath I. The pathogenesis of Burkitt's lymphoma. In Vande Woude GF, Klein G, eds. *Advances in Cancer Research* New York, Academic Press, 1990, **55**, 134.
18. Scheffner M, Munger K, Byrne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 1991, **88**, 5523–5527.
19. Crook T, Wrede D, Tidy JA, Mason WP, Evans DJ, Vousden, KH. Clonal p53 mutation in primary cervical cancer: association with human papillomavirus-negative tumours. *Lancet* 1992, **339**, 1070–1073.
20. Crook T, Vousden KH. Properties of p53 mutations detected in primary and secondary cervical cancers suggest mechanisms of metastasis and involvement of environmental carcinogens. *EMBO J* 1992, **11**, 3935–3940.

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