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Immunohistochemical Expression of Epstein–Barr Virus-encoded Latent Membrane Protein (LMP-1) in Paraffin Sections of EBV-associated Nasopharyngeal Carcinoma in Spanish Patients

F.J. Vera-Sempere,[1] J.S. Burgos,[1] M.S. Botella,[1] J. Cordoba[2] and M. Gobernado[2]

[1]Service of Pathology II, Laboratory of Molecular Pathology; and [2]Service of Microbiology and Virology, University Hospital La Fe, Medical School of Valencia University, Valencia 46009, Spain

Nasopharyngeal carcinoma (NPC) paraffin samples, from Spanish patients, of distinct histological types, including squamous cell carcinoma (10 cases), nonkeratinising carcinoma (12 cases) and undifferentiated carcinoma (29 cases) were analysed for Epstein–Barr virus (EBV) detection and EBV-encoded latent membrane protein (LMP-1) expression using a sensitive nested-polymerase chain reaction with four oligonucleotide primers specific for EBV genome (EB-1, 2, 3, 4) and immunohistochemistry by means of CS1–4 pool monoclonal antibody. EBV genome was detected regardless of histological type in 100% of samples with sufficient DNA quality to permit viral diagnosis (50 out of 51 cases), supporting the previous view that all types of NPC are variants of an EBV-associated malignancy. However LMP-1, an EBV-encoded oncogenic protein, was detected in 40 out of 51 samples (78.4%) and LMP-1 immunohistochemical expression was not apparently influenced by histological type, primary or metastatic site, clinical stage, age or sex. This high percentage of detection of LMP-1 in our cases supports a role for EBV in the pathogenesis of different types of NPC, but the lack of constant expression of LMP-1 in NPC remains unclear and various reasons are postulated to explain the absence of this oncogenic protein in some EBV-associated NPCs. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

The Epstein–Barr virus (EBV) is a member of the herpesvirus family which infects approximately 90% of the world's adult population. Primary infection usually occurs at an early age, is clinically dormant and results in life-long virus persistence. If EBV-infection is delayed, the self-limiting lymphoproliferative disorder known as infectious mononucleosis often ensues [1].

The virus displays a tropism for B lymphocytes and oropharyngeal epithelial cells [2, 3] across the complement receptor CD21, also known as CR2, and probably also by means of polymeric IgA-mediated viral entry [4]. In line with these tropisms EBV has been associated with several tumours, including polyclonal B lymphoproliferations in immunosuppressed individuals, Burkitt's lymphoma and, most recently, Hodgkin's disease, above all the mixed cellularity type [5]. However, the malignancy showing the most consistent association with the virus is probably nasopharyngeal carcinoma (NPC), and in fact EBV is detectable in virtually all cases of undifferentiated NPC worldwide [6].

The repertory of laboratory studies that can be used to detect EBV infection is limited, and diagnosis of associated diseases is often based on serological evidence [7], although, because EBV is virtually ubiquitous in adults, serological studies are of limited use in demonstrating a specific association of EBV with neoplasia. However, in the last decade polymerase chain reaction (PCR) technology has provided specific, rapid and sensitive means for the detection of viral genomes; furthermore, it is readily applied to paraffin-embedded archival tissues [8]. On the other hand, latent viral infection is associated with several different patterns of viral gene expression in infected cells that have contributed substantially to the recent identification of EBV-associated diseases. One of these latent viral products is the so-called "latent membrane protein" (LMP-1), an integral membrane protein, containing 386 amino acids, encoded by the *BNLF-1* gene (also called *LMP-1* gene) of EBV [9]. LMP-1 is an oncogenic protein related to cellular transformation processes

Correspondence to F.J. Vera-Sempere.
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and is defined as a viral oncogene because of its capacity to transform rodent fibroblasts and to render them tumorigenic in nude mice [10].

In this study we investigated the immunohistochemical expression of LMP-1 using a monoclonal anti-LMP-1 antibody pool (CS1–4) [11] in a series of cases of nasopharyngeal carcinoma (NPC) affecting Spanish people, which likewise have been screened for the presence of EBV-DNA by means of sensitive nested-PCR.

MATERIALS AND METHODS

Patients and tissue samples

Biopsy specimens were collected from 52 Spanish patients, age range from 15 to 79 years (mean 49.3 years), 32 males and 20 females (a ratio of 1:1.6), with histologically confirmed NPC. All patients were diagnosed, treated and followed up between 1977 and 1994 at University Hospital La Fe. Clinical and pathological aspects, such as age, sex, histological diagnosis, tumour stage, therapeutic procedures and survival time were abstracted from surgical pathology reports and clinical records. All biopsy tissues were fixed in formalin, embedded in paraffin, and examined by routine histological techniques, and all diagnoses of NPC were later confirmed by immunohistology with cytokeratin-specific monoclonal antibodies. Histopathological type was established by its most outstanding histological feature according to the WHO classification [12] and the clinical stages were graded according to the UICC staging classification of nasopharynx tumours [13]. Paraffin blocks of primary tumours were available from 44 patients and paraffin-embedded tissue of cervical lymph-node metastasis was available from 7 patients. Moreover, in one case tissue samples of non-tumoral nasopharyngeal mucosa adjacent to the tumour were also available for immunohistology and EBV-DNA screening.

Immunohistochemistry

Serial sections of tissues were stained with anti-latent membrane protein (LMP-1) [11], using a pool of four anti-LMP-1 monoclonal antibodies (Dako-EBV, CS 1–4, Code No. M897, Glostrup, Denmark) all directed against the internal part of the EBV-encoded latent membrane protein and reactive with at least three different epitopes of LMP-1. The staining with anti-LMP-1 was carried out on paraffin sections by use of biotinylated rabbit anti-mouse immunoglobulins and of avidin biotinylated horseradish peroxidase complex (ABC complex/HPR) (Dako, Denmark) [14]. The monoclonal anti-LMP-1 and biotinylated rabbit anti-mouse were used at dilutions of 1/40 and 1/200, respectively. All immunohistological sections were weakly counterstained to emphasise the absence of nonspecific immunostaining. Negative controls were performed in every case by omitting the primary and secondary antibodies, respectively, from the immunohistochemical procedure. As a positive control we used a well-known case of Hodgkin's disease of mixed cellularity with specific immunostaining of Reed–Sternberg cells [15].

EBV-DNA detection

Genomic DNA was extracted from paraffin-embedded tissues following standard procedures [16]. Cross-contamination of samples and false-positive PCRs were carefully avoided by frequent changing of gloves, use of positive displacement pipettes and strict spatial separation of the three main PCR steps (preparation of reaction mix, addition of target and amplification and manipulation of PCR products). DNA presence was verified by amplification of the β-globin gene, with the primers GH20 and PC04 [17]. A 268-bp fragment was obtained and detected after electrophoresis, when DNA extraction was positive. This presence was also verified and quantified by spectrophotometric analysis absorbance at 260 nm. EBV-DNA detection was carried-out with the nested-PCR technique. The DNA concentration used was 0.1–0.5 μg per 100 μl. We employed two primer sets (Table 1) that amplified a 297 bp or a 209 bp fragment from the *EBNA-1* gene [18]. Amplification was carried out in two successive rounds. The total reaction contained 50 mmol/l potassium chloride, 10 mmol/l Tris–HCl pH 8.4, 2.5 mmol/l magnesium chloride, 400 μmol/l of each dNTP, 0.15 μmol/l of primers EB-3 and EB-4 in the first round and 0.30 μmol/l of primers EB-1 and EB-2 in the second round, as well as 1 unit of ampliTaq DNA polymerase (Roche Molecular System, Inc., Brachburg, New Jersey, U.S.A.). The final reaction volume in each round was 100 μl. The first round was composed of one cycle of 95°C (3 min), 20 cycles of 95°C (1 min)–55°C (1 min)–72°C (1 min) and one cycle of 72°C (10 min). The second round consisted of one cycle of 95°C (3 min), 30 cycles of 95°C (1 min)–60°C (1 min)–72°C (1 min) and one cycle of 72°C (10 min). PCR was performed in an automated thermal cycler (Perkin-Elmer Cetus). After the first amplification with the EB-3 and EB-4 primers, 5 μl were transferred from the first to the second reaction mixture. After the second amplification with the EB-1 and EB-2

Table 1. Oligonucleotide primers for the polymerase chain reaction

Target	Primer	Sequence (5′→3′)	Base location*
	Outer†		
	EB-3	5′-AAG-GAG-GGT-GGT-TTG-GAA-AG-3′	109 332–109 351
	EB-4	5′-AGA-CAA-TGG-ACT-CCC-TTA-GC-3′	109 609–109 628
EBV	Inner‡		
	EB-1	5′-ATC-GTG-GTC-AAG-CAG-GTT-CC-3′	109 353–109 372
	EB-2	5′-ACT-CAA-TGG-TGT-AAG-ACG-AC-3′	109 542–109 561
Human	β_1 (GH20)	5′-GAA-GAG-CCA-AGC-ACA-GGT-AC-3′	
β-Globin§	β_2 (PC04)	5′-CCA-CTT-CAT-CCA-CGT-TCA-CC-3′	

*Base location in the EBV genome (strain B-95-8). †Product amplified size 297 pb. ‡Product amplified size 209 pb. §Product amplified size 268 pb.

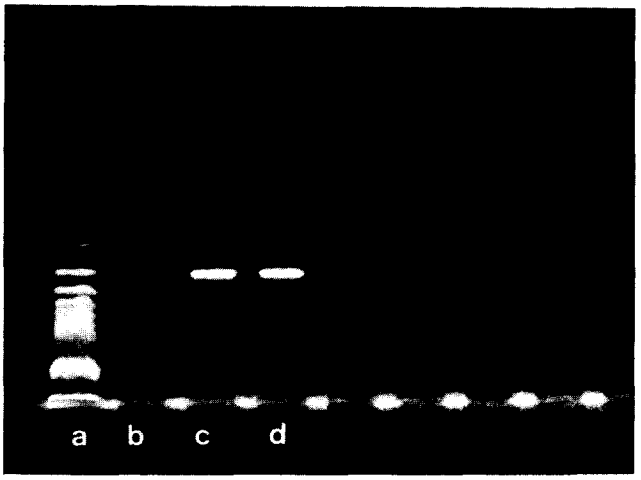

Fig. 1. Gel electrophoresis of amplified products after nested EBV PCR showing a band corresponding to product amplified size of 209 bp. Lane a: molecular mass marker; lane b: negative EBV control; lanes c and d: NPC cases.

primers, 17 μl of the amplified product from the second mixture were electrophoresed in a 2% agarose gel containing 0.5 μg/ml ethidium bromide. The resulting gel was photographed under ultraviolet light and regarded as positive when a band corresponding to the 209 bp fragment was present. The specificity of the amplified fragment visualised on the agarose gel was confirmed by a non-radioactive hybridisation assay (DNA enzyme immunoassay, GEN-ETI-K-DEIA, Sorin Biomedica, Saluggia, Italy). Furthermore, negative controls included different amplification products from different target DNAs (cytomegalovirus, human immunodeficiency virus and hepatitis B virus) with negative results.

For each amplification series, two types of controls were used: as an amplification positive control a case of post-transplant lymphoma (data not shown) studied previously as an EBV carrier (in this case, a 209 bp band appeared after electrophoresis); as a negative control normal samples from healthy individuals negative for EBV-DNA and sterile distilled water were used. All samples were tested at least twice in different experiments and the samples considered positive either in the case of concordant results or when a positive result could be confirmed by a subsequent experiment.

RESULTS

Primary NPC samples were histologically classified, according to the WHO scheme, as keratinising squamous cell carcinoma (SCC) (WHO type 1), non-keratinising squamous cell carcinoma (NKC) (WHO type 2) and undifferentiated carcinoma (UC) (WHO type 3) in 10, 12 and 29 cases, respectively. Likewise, seven metastatic lymph node samples were classified as metastatic undifferentiated carcinomas (UC) (6 cases) and metastatic non-keratinising carcinoma (one case). In addition, one tissue sample of non-tumoral nasopharyngeal mucosa adjacent to a UC-NPC was also available for immunohistochemical and molecular study.

Immunohistochemical analysis using monoclonal pool CS1–4 antibody (Table 2) revealed an overall LMP-1 positive staining in 40 out of 51 samples (78.4%) of NPC, so that in all histological NPC types there are frequent cases with LMP-1 immunohistochemical reactivity (Figs 2A–C). No significant differences were found when comparing immunohistochemical LMP-1 positivity in primary (35 out of 45 cases) (77.7%) and metastatic NPC samples (5 out of 7 cases) (71.4%). Likewise, immunohistochemical results showed no specific correlation between the expression of LMP-1 and histological type of NPC (primary and metastatic cases) so that approximately 80% (8 out of 10 cases), 58% (7 out of 12 cases) and 86% (25 out of 29 cases), respectively, of SCC, NKC and UC-NPC proved LMP-1 positive.

NPC cases with LMP-1 reactivity showed immunostaining only at neoplastic cell level and positivity was found with a predominant cytoplasmic pattern (Figs 2A–C), although occasionally the positivity appeared both on the surface—with a weak membranous pattern—and within the cytoplasm, even though the tumour cells were often not homogeneously labelled, and showed a patchy staining pattern. Background staining was either very weak or absent, especially if we consider that all immunohistological sections were weakly counterstained to emphasise the absence of nonspecific immunostaining. Infiltrating small lymphocytes, desmoplastic stromal reaction and overlying, histologically normal, epithelium present in primary samples, as well as one normal nasopharyngeal mucosae sample, were LMP-1 negative.

DNA from paraffin of all samples used for the immunohistochemical study was also extracted and PCRs run for β-globin and EBV-DNA. A total of 51 samples from NPC and one normal nasopharyngeal mucosae sample adjacent to NPC were available for amplification, whereas in only one case was no β-globin specific product obtained. In successful cases, extraction of human DNA from the samples was confirmed by amplification of the β-globin gene, and EBV-DNA was detected in 100% of these samples with sufficient quality to permit viral diagnosis (Fig. 1). In our study EBV-DNA positivity and LMP-1 expression were not influenced by histological type, clinical stage, age or sex. The pattern of LMP-1 staining in paraffin-processed NPC samples is correlated with EBV-DNA detection by nested-PCR in Table 2.

DISCUSSION

In the present study two different techniques for detecting EBV in NPC were employed: immunohistochemistry for detection of latent membrane protein (LMP-1) and nested-PCR using four oligonucleotide primers specific for EBV genome.

In our study, detection of EBV in NPC was made possible by a highly sensitive nested-PCR technique, and in 50 out of 51 Spanish NPC samples we were able to detect the presence of EBV-genome. In this sense it is well known that DNA amplification from fixed material varies depending on source, length of time in formalin and other less-studied factors [19], and in fact the only remaining EBV-negative NPC case in our series also failed to demonstrate amplification of the β-globin gene when paraffin-embedded tissue was employed. Likewise, we detected EBV-DNA in one normal nasopharyngeal sample adjacent to the NPC area analysed by nested-PCR.

Frequency of EBV-DNA positive nasopharyngeal carcinoma in the Spanish population has not been reported previously, although the Spanish Mediterranean coast probably represents a geographical area of intermediate risk [20] for the development of this malignancy. Our results indicate that EBV-DNA is uniformly associated with Spanish NPC cases, as hitherto reported for patients from high-risk areas

Table 2. Detection of LMP-1 protein and EBV-DNA in paraffin-embedded samples

Histological type	No. of samples	No. of β-globin DNA + samples	No. of EBV-DNA + samples	LMP-1 staining* (No. of samples) –	+	++	+++	No. of LMP-1 + samples
Primary NPC	44	43	43	9	12	11	12	35 (79.5%)
UC	23	22	22	3	7	6	7	20 (87.0%)
NKC	11	11	11	4	2	3	2	7 (63.6%)
SCC	10	10	10	2	3	2	3	8 (80.0%)
Secondary NPC	7	7	7	2	2	3	0	5 (71.4%)
UC	6	6	6	1	2	3	0	5 (83.3%)
NKC	1	1	1	1	0	0	0	0 (0.0%)
SCC	—	—	—	—	—	—	—	—
Total NPC	51	50	50	11	14	14	12	40 (78.4%)
Normal mucosae	1	1	1	1	0	0	0	0 (0.0%)

*–, negative staining; +, weak LMP-1 staining; ++, strong LMP-1 staining; +++, very strong LMP-1 staining.

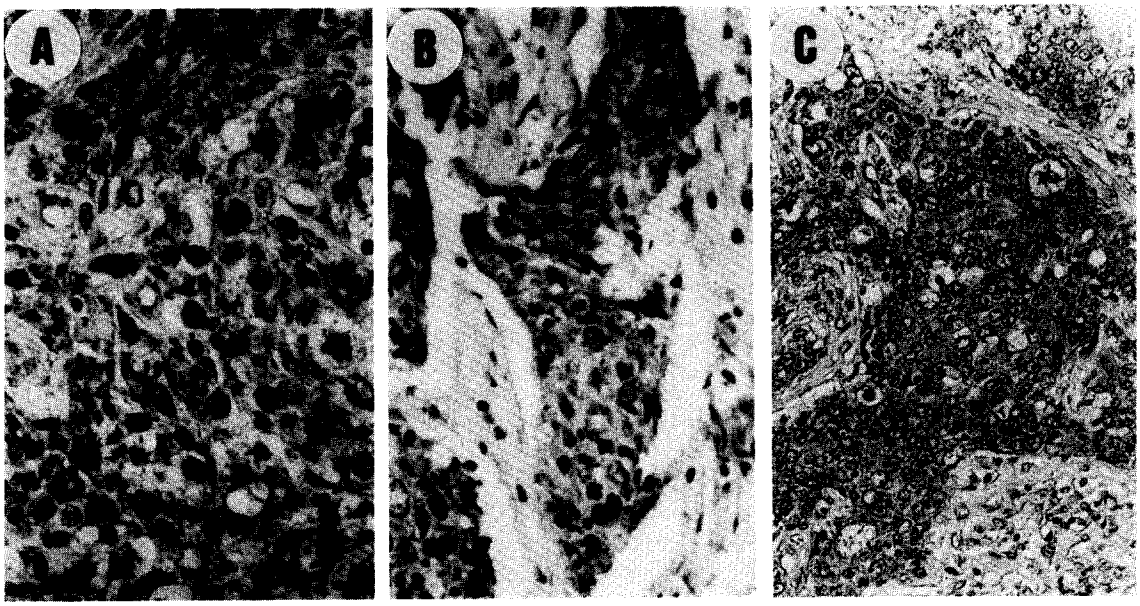

Fig. 2. Immunohistochemical staining for LMP-1 in NPC using CS1–4. (A) Undifferentiated carcinoma (UC) (WHO type 3) shows apparent and not homogeneous cytoplasmic positivity (400 ×). (B) Non-keratinising carcinoma (NKC) (WHO type 2) showing cords of tumour cells with LMP-1 cytoplasmic immunoreactivity. The intervening clear areas are fibrous septae negative for LMP-1 immunostaining (400 ×). (C) Moderately differentiated squamous cell carcinoma (SCC) (WHO type 1) showing cytoplasmic and less membranous immunoreactivity against LMP-1. A desmoplastic stromal reaction with scanty lymphocytes negative for LMP-1 is present (250 ×). All immunohistological sections were weakly counterstained to emphasise the absence of nonspecific immunostaining.

[21]. In our study, this association appears independent of the different histological NPC types, and this finding is in line with previous studies that demonstrated EBV-DNA in certain [21–23] or in all SCC cases [24], although with a low number of genome copies that probably are always within the limits of detection by nested-PCR technology (four EBV genomes by cell) at a difference of a single PCR assay, with either outer or inner primers, that need 400 genomes to detect EBV [18]. This varying sensitivity of the molecular technology used probably explains the differences in the percentage of SCC-NPC positives to EBV previously reported. In this way the first molecular studies by filter hybridisation showed absence of detectable EBV-DNA in SCC with various degrees of differentiation, while a Southern blot hybridisation study [6] and a PCR analysis of EBV transcription [24] showed that SCC, as with UC, contained EBV-DNA, although the viral genome copy was high in UC but low in SCC. A recent report in Italian patients with NPC using conventional PCR analysis showed that only a minority of SCC cases present EBV and always in a low copy number [25]. This low copy number of EBV-DNA detected by nested-PCR as used in our study, confirms the strong association between EBV and NPC in all histological types, although EBV probably plays a different oncogenic role in SCC than in UC, and also the geographic or ethnic origin of the patients may be important in determining the EBV association with NPC.

On the other hand, our study shows that 40 out of 51 samples (78.4%) of primary and metastatic NPC were positive for LMP-1 using a sensitive ABC immuno-

histochemical method. LMP-1 is an EBV latency protein implicated in oncogenesis, both *in vitro* and *in vivo* [10]. The high percentage of detection of LMP-1 in our samples, independent of histological type or primary/metastatic character, supports a role for EBV in the pathogenesis of different types of NPC. Failure to detect the protein in each specimen may reflect biological heterogeneity, or it could be due to limitations of this technique in paraffin-embedded tissues, as has been reported by others [15, 26]. Nevertheless our results are very similar to the positive level of LMP-1 found in NPC using the Western blot method [27, 28]. Nonetheless, earlier immunohistochemical studies reported LMP-1 positivity in tumour cells in only 22–30% of undifferentiated NPC when frozen tissue was used [29], whereas two other studies reported 50% [30] and 72% [24], respectively, for positivity with findings very close to ours. These different and contrasting results may be due to differences in technique. We employed an avidin–biotin–peroxidase complex technique that has been shown to be more sensitive than several other techniques, but the results presented here show clearly that only a proportion of NPC (not related to histological type or primary/metastatic location) expresses LMP-1 and therefore LMP-1 is probably expressed only in some unknown stages of NPC development [31]. Another possible reason for this great variety of results concerns the existence of various subsets of LMP-1 with different transformation abilities [31, 32]. Similarly, the variable expression of LMP-1 can be related to the methylation phenomenon of the LMP genome [33] that plays an important role in the regulation of this gene in NPC tumours. To date, it is not known why not all EBV-positive NPC express LMP-1. Probably technical reasons, such as the type and time of fixation, and immunohistochemical procedures, might explain why not all NPCs are stained, but we believe that further study using CS1–4 as well as other monoclonal antibodies against different epitopes of LMP-1 (S12 and anti-136) [27, 34] are indicated to determine the exact significance of variable LMP-1 immunohistochemical expression in EBV-associated NPC. New molecular and immunohistochemical studies in this sense are now in progress.

1. Liebowitz D. Nasopharyngeal carcinoma: the Epstein–Barr virus association. *Semin Oncol* 1994, **21**, 376–381.
2. Fingeroth JD, Weiss JJ, Tedder TF, *et al.* Epstein–Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci USA* 1984, **81**, 4510–4514.
3. Sixbey JW, Nedrud JG, Raab-Traub N, *et al.* Epstein–Barr virus replication in oropharyngeal epithelial cells. *N Engl J Med* 1984, **310**, 1225–1230.
4. Sixbey JW, Yao O. Immunoglobulin A-induce shift of Epstein–Barr virus tissue tropism. *Science* 1992, **255**, 1578–1580.
5. Niedobitek G, Young LS. Epstein–Barr virus persistence and virus-associated tumours. *Lancet* 1994, **343**, 333–335.
6. Raab-Traub N, Flynn K, Pearson G, *et al.* The differentiated form of nasopharyngeal carcinoma contains Epstein–Barr virus DNA. *Int J Cancer* 1987, **39**, 25–29.
7. Pearson GB, Weiland L, Neel HB III, *et al.* Application of Epstein–Barr virus (EBV) serology to the diagnosis of North American nasopharyngeal carcinomas. *Cancer* 1983, **51**, 260–268.
8. Ambinder RF, Mann RB. Detection and characterization of Epstein–Barr virus in clinical specimens. *Am J Pathol* 1994, **145**, 239–252.
9. Hudson GS, Farrel PJ, Barrell BG. Two related but differentially expressed potential membrane proteins encoded by the *EcoRI* Dhet region of Epstein–Barr virus B95–8. *J Virol* 1985, **53**, 528–535.
10. Wang D, Leibowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* 1985, **43**, 831–840.
11. Rowe M, Evans HS, Young LS, Hennesy K, Kieff K, Rickinson AB. Monoclonal antibodies to the latent membrane protein of Epstein–Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. *J Gen Virol* 1987, **68**, 1575–1586.
12. Shanmugaratnam K, Sobin LH. *Histological Typing of Upper Respiratory Tract Tumours*, International Histological Classification of Tumours, No. 19. Geneva: WHO, 1978.
13. U.I.C.C. *TNM Atlas. Illustrated Guide to the TNM/pTNM Classification of Malignant Tumours.* 3rd Edn, 2nd revision. Berlin, Springer, 1992.
14. Wood GS, Warnke R. Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin–avidin detection systems. *J Histochem Cytochem* 1981, **29**, 1196–1204.
15. Lauritzen AF, Hording U, Nielsen HW. Epstein–Barr virus and Hodgkin's disease. A comparative immunological, *in situ* hybridization and polymerase chain reaction study. *APMIS* 1994, **102**, 495–500.
16. Maniatis T, Fritsch EF, Sambrook J. *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1982.
17. Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). *PCR Protocols. A Guide to Methods and Applications.* San Diego, CA, Academic Press, 1990.
18. Cinque P, Brytting M, Vago L, *et al.* Epstein–Barr virus DNA in cerebrospinal fluid from patients with AIDS-related primary lymphoma of the central nervous system. *Lancet* 1993, **342**, 398–401.
19. Telenti A, Marshall WF, Smith TF. Detection of Epstein–Barr virus by polymerase chain reaction. *J Clin Microbiol* 1990, **28**, 2187–2190.
20. Hubbert A, De The G. Comportement alimentaire, modes de vie et cancer de rhinopharynx. *Bull Cancer* 1982, **69**, 476–482.
21. Dickens P, Srivastava G, Loke SL, Chan CW, Liu YT. Epstein–Barr virus DNA in nasopharyngeal carcinomas from Chinese patients in Hong Kong. *J Clin Pathol* 1992, **45**, 396–397.
22. Yeung WM, Zong YS, Chiu CT, *et al.* Epstein–Barr virus carriage by nasopharyngeal carcinoma *in situ*. *Int J Cancer* 1993, **53**, 746–750.
23. Hørding U, Nielsen HW, Albeck H, Daugard S. Nasopharyngeal carcinoma: histopathological types and association with Epstein–Barr virus. *Oral Oncol, Eur J Cancer* 1993, **29B**, 137–139.
24. Pathmanathan R, Prasad U, Chandrika G, Sadler R, Flynn K, Raab-Traub N. Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein–Barr virus-infected neoplasia. *Am J Pathol* 1995, **146**, 1355–1367.
25. Della Torre G, Pilotti S, Donghi R, *et al.* Epstein–Barr virus genomes in undifferentiated and squamous cell nasopharyngeal carcinomas in Italian patients. *Diagn Mol Pathol* 1994, **3**, 32–37.
26. Pallensen G, Hamilton-Dutoit SJ, Rowe M, Young LS. Expression of Epstein–Barr virus latent gene products in tumour cells of Hodgkin's disease. *Lancet* 1991, **337**, 320–322.
27. Fåhraeus R, Fu HL, Ernberg I, *et al.* Expression of Epstein–Barr virus-encoded proteins in nasopharyngeal carcinoma. *Int J Cancer* 1988, **42**, 329–338.
28. Young LS, Dawson CW, Clark D, *et al.* Epstein–Barr virus gene expression in nasopharyngeal carcinoma. *J Gen Virol* 1988, **69**, 1051–1065.
29. Niedobitek G, Young LS, Sam CK, Brooks L, Prasad U, Rickinson AB. Expression of Epstein–Barr virus genes and of lymphocyte activation molecules in undifferentiated nasopharyngeal carcinomas. *Am J Pathol* 1992, **140**, 879–887.
30. Zheng X, Hu L, Chen F, Christensson B. Expression of Ki 67 antigen, epidermal growth factor receptor and Epstein–Barr virus-encoded latent membrane protein (LMP-1) in nasopharyngeal carcinoma. *Oral Oncol, Eur J Cancer* 1994, **30B**, 290–295.
31. Hu LF, Chen F, Ernberg I, *et al.* Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP-1. *Oncogene* 1993, **8**, 1575–1583.
32. Trivedi P, Hu LF, Chen F, *et al.* Epstein–Barr(EBV)-encoded membrane protein LMP-1 from a nasopharyngeal carcinoma is

non-immunogenic in a murine model system, in contrast to a B cell-derived homologue. *Eur J Cancer* 1994, **30A**, 84–88.
33. Hu LF, Minarovits J, Shi-Long C, *et al.* 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* 1991, **65**, 1558–1567.
34. Jiwa NM, Oudejans JJ, Dukers DF, *et al.* Immunohistochemical demonstration of different latent membrane proteins-1 epitopes of Epstein–Barr virus in lymphoproliferative diseases. *J Clin Pathol* 1995, **48**, 438–442.

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