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ORIGINAL PAPER

Epstein-Barr Virus Latent Membrane Protein 1 is not Associated with Vessel Density nor with Hypoxia Inducible Factor 1 Alpha Expression in Nasopharyngeal Carcinoma Tissue

Alexander A. Benders · Weihua Tang · Jaap M. Middeldorp · Astrid E. Greijer · Leigh B. Thorne · William K. Funkhouser · W. Kimryn Rathmell · Margaret L. Gulley

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Abstract Hypoxia-inducible factor-1α (HIF-1α) and the neo-angiogenic factors induced as a result of hypoxia-inducible factor transcriptional activation may contribute to tumorigenesis by inducing vessel formation that in turn provides oxygen and nutrients promoting tumor expansion. In vitro studies of nasopharyngeal carcinoma (NPC), an aggressive malignancy that is nearly always infected by Epstein–Barr virus, show HIF-1α is upregulated by viral latent membrane protein 1 (LMP1). The current study used immunohistochemistry to examine the extent to which HIF-1α and LMP1 are co-expressed in naturally infected NPC tissues. Analytic procedures were optimized for sensitive localization of HIF-1α and LMP1 in fixed tissue sections using immunohistochemistry with sensitive fluorescent and signal amplification technologies. Vessel density was quantified by CD31 immunohistochemistry. LMP1 was expressed focally in all 18 NPCs examined, including 7/8 in situ lesions. There was no consistent co-localization with HIF-1α which was usually only weakly expressed in a subset of neoplastic cells. Neither LMP1 nor HIF-1α expression correlated with vessel density, and degree of vascularization varied widely among cases. Advanced immunohistochemical technologies reveal that LMP1 is expressed more commonly than previously reported in NPC. There is no consistent relationship between LMP1 and either HIF-1α expression or degree of microvasculature. The biologic basis for the wide variation in vessel density deserves further investigation.

Keywords Nasopharyngeal carcinoma · HIF-1α · Angiogenesis · Immunohistochemistry

A. A. Benders · W. Tang · L. B. Thorne · W. K. Funkhouser · M. L. Gulley (✉)
Department of Pathology and Laboratory Medicine, The Lineberger Comprehensive Cancer Center, University of North Carolina, 101 Manning Dr, 913 Brinkhous-Bullitt Building, Chapel Hill, NC 27599-7525, USA
e-mail: margaret_gulley@med.unc.edu

A. A. Benders · J. M. Middeldorp · A. E. Greijer
Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

W. Kimryn Rathmell
Departments of Medicine and Genetics, The Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

Introduction

Neoplasms frequently induce formation of new blood vessels, supplying nutrients and oxygen that facilitate cell growth and invasion. Insufficient neo-angiogenesis may result in tumor necrosis, and a potential strategy for treating cancer is to prevent new vessel formation using pharmacologic means. Angiogenic blockers are now used therapeutically in renal cell carcinoma and in HHV8-related Kaposi's sarcoma in which the virus is thought to drive vascularization via VEGF [1, 2].

Epstein-Barr virus (EBV) is strongly associated with nasopharyngeal carcinoma (NPC) as evidenced by the presence of EBV DNA within the malignant cells in the majority of cases [3, 4]. EBV infection and neo-angiogenesis in NPC has been previously explored by counting microvessel density and by immunohistochemical analysis of vascular endothelial growth factor (VEGF) expression, and it was found that vessel density and VEGF expression were higher in NPC compared to normal nasopharyngeal tissue [5]. In addition, there was significantly more invasion and metastasis in EBV-infected tumors compared to

EBV-negative carcinomas, particularly in older patients [6]. This could be explained, at least in part, by modulation of signaling cascades by EBV in response to hypoxia, as suggested by Lo et al. [7].

Angiogenesis is mediated by a series of proteins beginning with hypoxia-inducible factors 1α and 2α (HIF-1α and HIF-2α) which are transcription factors that respond to changes in available oxygen in the cellular environment. HIF-1α in turn, affects transcription of genes encoding VEGF, carbonic anhydrase IX (CAIX), and matrix metalloproteinase 9 (MMP9) among a transcriptional repertoire of dozens of targets mediating neo-angiogenesis and metastasis [8]. An immunohistochemical study by Hui et al. [9] found poorer survival among NPC patients whose tumors had high HIF-1α expression. The link between HIF-1α expression and survival has also been reported in other cancers including breast cancer [10, 11].

Latent Membrane Protein 1 (LMP1) is considered to be among the most likely EBV proteins to contribute to oncogenesis and tumor maintenance [12, 13]. A relation between LMP1 expression and increased HIF-1α expression was found by Wakisaka et al. [14] in both lymphoblastoid and epithelial cell lines. Subsequently Kondo et al. [15] showed the mechanism by which LMP1 functions—it stabilizes SIAH1, an E3 ubiquitin ligase, which in turn ubiquitinates and destabilizes prolyl hydroxylase proteins (PHD1 and PHD3) that, under normoxic conditions, are responsible for hydroxylation and subsequent degradation of HIF-1α. In this manner, LMP1 expression effectively increases HIF-1α levels leading to downstream transcription of angiogenic factors.

The current study determines the extent to which LMP1 and HIF-1α expression are associated in naturally infected NPC tissues. Co-localization of LMP1 and HIF-1α would imply that the same biochemical pathways that are operative in cell line models are also operative in vivo, potentially contributing to angiogenesis, tumor growth, and propensity to metastasis in EBV-infected cancer patients. HIF-1α was detected by immunohistochemical analysis of formalin fixed paraffin-embedded biopsy tissue. Chromogenic and immunofluorescent histochemical approaches were compared to optimize sensitive detection of LMP1 expression, and CD31 was used to assist in morphologic quantification of vascularity.

Materials and Methods

Case Selection

Formalin-fixed, paraffin-embedded tissues from 21 NPC patients were retrieved from the archives of the Gad-jah Madah University, Yogyakarta, Indonesia ($n = 12$ originating from Indonesian patients) and from the University of North Carolina at Chapel Hill ($n = 9$ originating from American patients). Some tumors were collected at initial diagnosis and others represented recurrence following therapy. Specimens were confirmed by a pathologist to represent NPC, and all tumors were previously documented to be EBV-related as shown by localization of EBV-encoded RNA (*EBER1*) and/or EBV nuclear antigen (EBNA1) to the malignant epithelial cells using the Ventana Benchmark ISH system (Tucson, AZ, USA) for *EBER1* detection in the American cases, and the PNA ISH kit with *EBER* probe (DAKO, Glostrup, Denmark) or immunostaining with EBNA1 OT1X monoclonal antibody for the Indonesian cases [16]. Three cases (two Indonesian, one American) were excluded because of insufficient tumor tissue to complete all analyses, resulting in a total of 18 cases.

Chromogenic Immunohistochemical Stains for LMP1, HIF-1α and CD31

Latent membrane protein 1 (LMP1) was targeted using the mouse monoclonal cocktail CS1-4 (M0897; DAKO) at a dilution of 1:100, while HIF-1α was detected using rabbit polyclonal antibody (sc-10790; SantaCruz, Santa Cruz, CA, USA) at 1:100, and CD31 was detected using mouse monoclonal anti-human CD31 (DAKO) at 1:100. Citrate antigen retrieval (Citra, BioGenex, San Ramon, CA, USA) was performed in a microwave for 30 min, endogenous peroxidase activity was blocked (DAKO peroxidase block solution) for 20 min, and protein blocking (BioGenex) was performed for 10 min. Sections were incubated in primary antibody overnight at 4°C followed by detection using Super Sensitive Polymer-HRP IHCX Detection system (Biogenex) with diaminobenzidine as the substrate. The sections then were counterstained with hematoxylin, dehydrated, and mounted. Control tissues included *EBER*-positive Hodgkin lymphoma for LMP1, and renal cell carcinoma tissue for HIF-1α and CD31. Additional controls were renal cell carcinoma cell line pellets (RCC4 2-1 over expresses HIF-1α while RCC4 3-14 does not) that were fixed in formalin and paraffin embedded [17]. Specificity was assured in all specimens by parallel stains omitting the primary antibody.

Immunofluorescent Histochemical Stains for LMP1

To maximize low level detection, LMP1 protein was additionally evaluated by immunofluorescence using filters to visualize the relevant bandwidth of the marker. Antigen retrieval remained the same as described above for chromogenic stains. LMP1 was targeted using the same antibody cocktail at 1:100 and results were compared with a higher dilution of 1:500 in combination with the Tyramide

Signal Amplification (TSA) kit (PerkinElmer, MA, USA) to enhance signal-to-noise ratio. Of note, DAKO's catalyzed signal amplification (CSA) system in combination with Direct Labeled Secondary anti-mouse (DAKO) was also tested and produced similar results. Sections were treated with biotin-labeled secondary goat antibody (PerkinElmer) and streptavidin horseradish peroxidase (SA-HRP, BioGenex). For the TSA system additional biotin and avidin blocking steps were implemented (BioGenex). Fluorophore Cyanine 3 (PerkinElmer) reacted with HRP to allow visualization of the signal by immunofluorescent microscopy, and DAPI counterstain revealed nuclear and architectural features to help distinguish benign from malignant cells.

Scoring of Chromogenic and Immunofluorescent Histochemical Stains

For LMP1, only cytoplasmic and membrane signals were evaluated, whereas for HIF-1α only nuclear signals were evaluated since nuclear HIF-1α is presumed to be the active form of the transcription factor. The proportion score (PS) for neoplastic cells was expressed as a score of 0–5 where 0 is no staining, 1 is 1 out of 100 cells, 2 is 1 out of 10 cells, 3 is 1 out of 3 cells, 4 is 2 out of 3 cells, and 5 is virtually all cells. The intensity score (IS) for neoplastic cells was expressed on a scale of 0–3 where 0 is no staining at all, 1 is weak staining, 2 is intermediate, and 3 is strong staining. When intensity varied within a given neoplasm, the average intensity was judged. Total score was the sum of the PS and IS. The pre-malignant lesions were considered too small for accurate scoring, so results were interpreted qualitatively as positive or negative for each marker. To score the number of vessels in a 40× field, 5 different regions of the neoplasm were evaluated on a CD31-stained slide, and number of vessels was averaged to obtain a score from 0 to 3 where 0 is no vessels, 1 is 1–19 vessels, 2 is 20–39 vessels and 3 is higher than 40 vessels per 40× field.

Results

In a series of 18 Indonesian and American NPCs, every case was confirmed to have invasive carcinoma, and 12 also had premalignant lesions while three had only normal squamous and/or columnar surface epithelium, and another three lacked any surface epithelial component. Since small vessels were often difficult to discern on H&E stained sections, CD31 immunostains targeting endothelial cells assisted in counting the number of vessels in the areas with invasive carcinoma. The amount of microvasculature varied widely among cases from very few to over 40 vessels per 40× field (Table 1).

LMP1 Expression

Chromogenic staining showed focal LMP1 expression in some but not all NPCs. Immunofluorescent staining of LMP1 on the same tissues revealed a greater proportion and intensity of positive signals in malignant cells, suggesting that the fluorescent method was more sensitive than the chromogenic method. Furthermore, including the tyramide signal amplification step as compared to not implementing this step yielded even more robust detection of LMP1 protein among viable tumor cells (Fig. 1). When tested using the most sensitive detection method (immunofluorescence with signal amplification), LMP1 was expressed in all 18 invasive NPCs, usually at weak to moderate intensity and in patterns ranging from rare individual cells to over half of all tumor cells.

Among the pre-malignant lesions, LMP1 was expressed in a fraction of cells of the surface epithelium representing histologies ranging from low grade dysplasia to carcinoma in situ, whereas normal epithelium had no LMP1 expression in benign surface components, even using the most sensitive detection method of tyramide signal amplification with immunofluorescent staining. This result implies that LMP1 is expressed only in neoplastic cells and not in normal epithelium. Overall, 7/8 patients with evaluable pre-malignant lesions had LMP1 expression in the surface component of their tumor (Table 2).

HIF-1α Expression and Associations with LMP1 and Vessel Density

Hypoxia-inducible factor-1α (HIF-1α) was expressed focally and weakly in the majority of invasive NPCs (15/18 cases, 83%; Table 1). HIF-1α and LMP1 expression patterns were not similar, nor was the intensity of expression of these two proteins correlated in specific tumors (Table 1). Dual immunostains were not feasible, but evaluation of staining patterns and proportion scores suggested that LMP1 and HIF-1α did not consistently co-localize. Likewise, there was no strong correlation between LMP1 scores and the degree of vascularization as measured with the assistance of CD31 immunostains, nor was there a strong association between HIF-1α scores and degree of vascularization, implying that factors beyond HIF-1α are likely to influence new vessel growth.

Among premalignant lesions, HIF-1α was often expressed in atypical as well as benign-appearing squamous epithelial cell nuclei (Table 2). Interestingly, case #16 had LMP1 and HIF-1α co-expression in basal layers of a mild dysplasia lesion in which cellular atypia was limited to the basal layer (Fig. 2). Expression was confirmed as true positive upon evaluation of the control slide in which omission of the primary antibody resulted in lack of signal.

Table 1 Expression of EBV LMP1 and HIF-1α, and vessel density in invasive nasopharyngeal carcinomas

Case #	Geographic origin	Vasculature, CD31 Density score	EBV LMP1*			HIF-1α		
			Proportion score	Intensity score	Total score	Proportion score	Intensity score	Total score
1	Indonesia	3	2	1	3	2	2	4
2	USA	3	2	1	3	1	1	2
3	Indonesia	3	1	2	3	1	1	2
4	Indonesia	2	3	2	5	1	1	2
5	USA	2	3	1	4	1	1	2
6	Indonesia	2	4	3	7	1	1	2
7	Indonesia	2	4	2	6	1	1	2
8	Indonesia	2	2	1	2	0	0	0
9	USA	1	3	2	5	1	1	2
10	Indonesia	1	1	2	3	1	1	2
11	USA	1	4	2	6	0	0	0
12	Indonesia	1	3	1	4	3	1	4
13	USA	1	3	1	4	0	0	0
14	Indonesia	1	1	1	2	1	1	2
15	Indonesia	1	1	2	3	1	1	2
16	USA	1	2	1	3	2	1	3
17	Indonesia	0	2	2	4	1	1	2
18	USA	0	2	1	3	2	1	3
Total positive		16/18	18/18	18/18	18/18	15/18	15/18	15/18

LMP1 latent membrane protein 1; *HIF-1α* hypoxia inducible factor 1 alpha; *USA* United States of America

* LMP1 scores represent results of immunofluorescent stains using tyramide signal amplification

Case #17 also co-expressed LMP1 and HIF-1α in basal cells. Although these two examples of coordinated expression were identified, the majority of cases did not appear to have similar patterns of expression.

Discussion

This study describes a sensitive method of detecting LMP1 using fluorescence and signal amplification resulting in identification of LMP1 expression in a much higher proportion of NPC cases than had been identified using traditional chromogenic immunohistochemistry with brightfield microscopy. While both LMP1 and HIF-1α were often expressed in invasive NPC tissues, there does not appear to be a consistent positional or quantitative relationship between LMP1 expression and HIF-1α upregulation within the invasive component of NPC. This implies that HIF-1α expression is controlled by factors beyond LMP1. Likewise, there was no strong correlation between HIF-1α expression and the degree of microvascularization, implying that factors beyond HIF-1α control vessel density. Finally, this study documents a similar distribution of microvasculature counts as well as LMP1 and HIF-1α expression between patient cohorts from Indonesia and the USA, suggesting no geographical bias.

Prior studies showed that LMP1 transfection of lymphoblastoid and carcinoma cell lines induced HIF-1α in vitro [14, 15]. If the same pathway were active in vivo, it might compel clinical trials of newly available anti-angiogenic agents to thwart the effects of LMP1. Anti-angiogenic agents are effective in treating clear cell carcinoma of the kidney where mutation of *VHL* with accompanying HIF induction is thought to drive tumor growth.

Compared to in vitro models in which LMP1 induced HIF-1α [14, 15], in vivo settings can be expected to behave in a much more complicated fashion. Levels of host genes are influenced, at least in part, by the local tumor environment including oxygenation status, which is a potent regulator of HIF stabilization. HIF proteins are very dynamic, with levels accommodating quickly to a wide variety of genetic and environmental stimuli. Indeed, the human genome has over 100 hypoxia response genes controlling angiogenesis, anaerobic metabolism, apoptosis, and erythrocyte production [18]. Even renal cell carcinomas harboring *VHL* mutation have variable levels of HIF-1α upregulation, and *VHL* mutant tumors expressing HIF-2α exclusively display a distinct transcriptional profile [19].

Recognizing that HIF-1α is only one of many factors through which LMP1 might influence angiogenesis, we also measured vessel density using CD31-assisted microscopy. A wide range of vessel counts was found but,

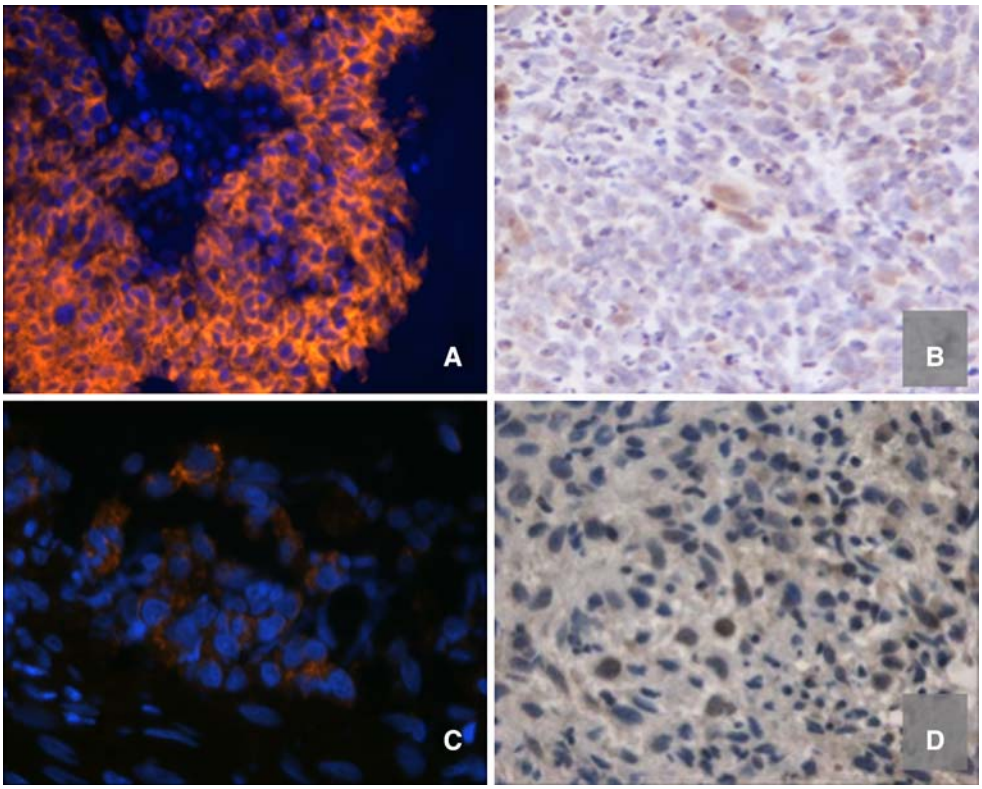

Fig. 1 In invasive nasopharyngeal carcinomas, intensity of LMP1 expression does not correlate with HIF-1α expression. *Panel A* (case 6) shows relatively diffuse, strong cytoplasmic LMP1 localized to malignant cells, while *panel B* (case 6) shows focal weak nuclear HIF-1α expression in large malignant-appearing cells. *Panel C* (case 12) shows focal weak LMP1 expression in cords of malignant cells, while *panel D* (case 12) shows nuclear HIF-1α expressed at varying intensity in large malignant-appearing cells [**a** and **c**: orange immunofluorescence with *blue* DAPI counterstain (200× and 400×, respectively); **b** and **d**: immunohistochemistry with *brown* signal and blue hematoxylin counterstain (400×)]

Table 2 Comparison of LMP1 and HIF-1α expression in invasive and in situ components of twelve nasopharyngeal carcinomas

Case #	Geographic origin	Invasive component		Pre-malignant component	
		EBV LMP1 Total score	HIF-1α Total score	EBV LMP1	HIF-1α
6	Indonesia	7	2	Positive	Positive
9	USA	5	2	N/A	Neg
4	Indonesia	5	2	Positive	N/A
5	USA	4	2	N/A	Positive
13	USA	4	0	Neg	N/A
17	Indonesia	4	2	Positive	Positive
18	USA	3	3	N/A	Positive
2	USA	3	2	N/A	Positive
3	Indonesia	3	2	Positive	Positive
1	Indonesia	3	4	Positive	Positive
15	Indonesia	3	2	Positive	Neg
16	USA	3	3	Positive	Positive

Neg negative result; *N/A* uninterpretable result; *HIF-1α* hypoxia inducible factor 1 alpha; *LMP1* latent membrane protein 1; *USA* United States of America

again, there was no obvious relationship between LMP1 expression scores and the degree of microvasculature. This does not exclude a relationship between EBV infection and angiogenesis since our sample size was small and we did not account for the fact that natural EBV infection introduces nearly 100 foreign coding sequences of which

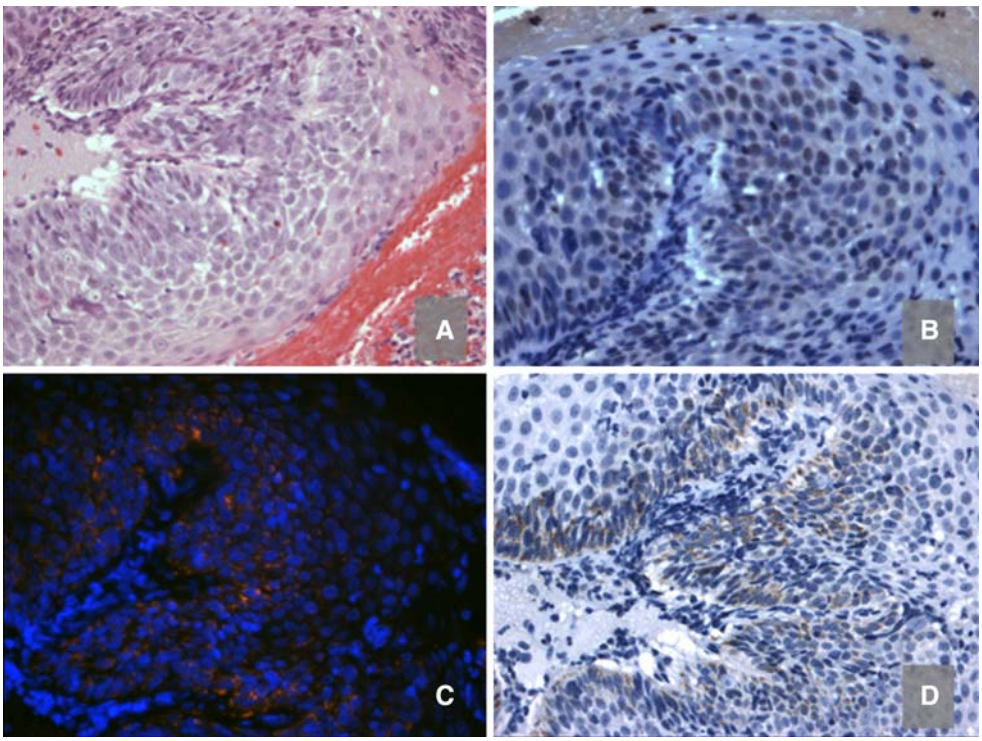

Fig. 2 HIF-1α and LMP1 are expressed in a premalignant lesion (case 16). H&E stain (**a**) reveals squamous epithelium with focal mild dysplasia, while HIF-1α immunohistochemistry (**b**) reveals HIF-1α protein in many epithelial cells throughout the thickness of the surface epithelium. *Panels C* and *D* demonstrate LMP1 expression concentrated in the basal layers by inmmunofluorescent and chromogenic methods, respectively (400×)

several beyond LMP1 are typically expressed, at least focally, in NPC. These include the viral gene products *EBER1*, *EBER2*, EBNA1, LMP2, BARF1 and several microRNAs [20–22].

According to Zhong et al. [23], HIF-1α upregulation is probably an early event in prostate carcinogenesis. It is feasible that HIF-1α is expressed transiently or is active only in specific stages of malignancy. Further evaluation of premalignant lesions is warranted given our anecdotal observation of LMP1 and HIF-1α co-expression in basal epithelial cells in two cases. Indeed, LMP1 is thought to have different biological properties at various stages of disease progression and may induce additional factors that contribute to neovascularisation [24–26].

This study focused on LMP1 because of the previously identified link between LMP1 and HIF-1α in cell lines, and because LMP1 is considered to be among the most oncogenic of the EBV proteins. Sensitive detection of LMP1 is important because it is feasible that even low level LMP1 may trigger downstream functions. Prior studies have shown wide differences in LMP1 positivity among NPC cohorts [6, 27–29]. This work demonstrates that detection of LMP1 expression depends on the sensitivity of the analytic methods that are used. When traditional chromogenic immunohistochemistry was used, less LMP1 expression was identified compared to when fluorescent detection was used. Sensitivity could be enhanced even further using signal amplification technologies. Research is underway to develop systems for measuring fluorescent signals in situ that will facilitate quantification of multiple factors in the same cells or lesions [30–32].

High numbers of microvessels in some of our NPC cases imply that microvasculature may be a critical factor in supporting tumor growth and might represent a potential target for intervention. Further work should be done to explore whether NPC patients benefit from anti-angiogenic therapies and to measure the extent to which laboratory evaluation of vessel density or upstream mediators predicts response to treatment [33, 34].

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