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Expression of Ki67 Antigen, Epidermal Growth Factor Receptor and Epstein–Barr Virus-encoded Latent Membrane Protein (LMP1) in Nasopharyngeal Carcinoma

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The expression of Ki67 antigen, epidermal growth factor receptor (EGFR) and the Epstein–Barr virus (EBV)-encoded latent membrane protein (LMP1) in nasopharyngeal carcinoma (NPC) was immunohistochemically determined. In cases with sufficient material western blot analysis was applied to analyse the LMP1 expression. Biopsies from 20 Chinese and 3 Swedish patients with NPC were included in the study. Our results demonstrated a nuclear Ki67 staining, a membrane EGFR staining, and a dot-like cytoplasmic and/or membrane LMP1 staining pattern in tumour cells of NPC. The proportion of Ki67-positive cells correlated with tumour stage. A strong expression of EGFR was frequently seen in patients with tumour stages III and IV and was paralleled by a higher proportion of Ki67-positive cells. The majority of the LMP1-positive cases strongly expressed EGFR and had a higher proportion of Ki67-positive cells, indicating a possible effect of EBV LMP1 on the proliferation of tumour cells in NPC. The increased expression of EGFR and Ki67 in NPC at late tumour stage indicates their possible use in malignancy grading of NPC.

Keywords: Ki67, EGFR, LMP1, NPC, immunohistochemistry

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INTRODUCTION

NASOPHARYNGEAL CARCINOMA (NPC) has unique epidemiological and biological characteristics. The incidence of NPC is high in southern China and is also increased in northern Africa compared to most other parts of the world [1]. Regardless of the geographic origin, NPC is consistently associated with Epstein–Barr virus (EBV)[2]. EBV genomes have been demonstrated in 100% of anaplastic NPC [2, 3]. Expression of the EBV-encoded protein in NPC is restricted to EBV nuclear protein (EBNA)1 and latent membrane protein (LMP)1[4]. EBNA1, binding to the origin of latent viral replication, is required for the maintenance of multiple viral genomes in episomal form [5]. LMP1, a membrane protein encoded from *Bam*H 1 fragment of the EBV genomes, contains 399 amino acids and is approximately 63–66 kD in size. Expression of LMP1 in established rodent fibroblast lines [6] and human keratinocyte lines [7, 8] is associated with *in vitro* transformation. Recently, the sequence of the LMP1 gene from the Cao tumour (nude mouse propagated tumour from Chinese NPC) was found to differ considerably from the corresponding gene in the B95.8 (marmoset B-cell line immortalised by EBV from a patient with infectious mononucleosis) isolate [9]. A Cao-LMP1-transfected human keratinocyte line showed a higher tumorigenicity than its B95.8-LMP1-transfected counterpart [10] indicating that the NPC-derived LMP1 gene more effectively predisposes the cells for a tumorigenic change. However, the precise role of EBV in the development of NPC is still poorly understood.

Analysis of tumour growth characteristics can provide insights into tumour cell biology that may allow the design of more effective tumour therapy. Proliferative activity analysed by different proliferation parameters such as mitotic counts, labelling index with tritiated thymidine incorporation and DNA flow cytometric analysis has been related to the degree of

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malignancy in various neoplasms [11]. Recently, monoclonal antibodies (MAb) that recognise cell cycle-associated molecules such as Ki67 and PCNA have been used to determine growth fractions in a number of tumours [12, 13].

Epidermal growth factor receptor (EGFR), a glycoprotein with a molecular weight of 170 kD, acts as a receptor for EGF-stimulated cell proliferation. Its expression has been described in many human neoplasms, such as brain [14], genitourinary [15], gynaecological [16], lung [17] and breast tumour [18] and is suggested to indicate a poor prognosis. In a recent study, Santini *et al.* applied a simplified competition radio ligand assay to analyse the EGFR level in head and neck squamous cell carcinoma including NPC [19]. Tumour EGFR levels were significantly linked to the size and the stage of the lesions, suggesting that EGFR measurement may be of prognostic value. However, the biochemical binding assay is not adequate to examine the localisation of EGFR in tissues.

In the present study, the expression of Ki67 and EGFR was immunohistochemically determined in normal nasopharyngeal epithelia and NPC. The expression of LMP1 in NPC was analysed by immunohistochemistry and western blot analysis. The purpose of the present study was: (1) to investigate the possible correlation between LMP1 expression and the proliferative activity of tumour cells as determined by EGFR and Ki67 in NPC, and (2) to compare these parameters with TNM and clinical stage of NPC to indicate their possible value as prognostic markers.

MATERIALS AND METHODS

Specimens

Biopsy specimens were collected from 20 Chinese and 3 Swedish patients with histologically confirmed poorly differentiated or undifferentiated NPC. Adenoids from adenoidectomy were used as source of normal nasopharyngeal epithelium. Histopathological grading was performed according to WHO classification [20]. The clinical stages of both the Chinese and Swedish patients were classified according to the staging classification of NPC defined in the Chinese National Conference of NPC [21].

Tumour samples were snap frozen immediately after surgical removal and kept at −70°C before use. Cryostat sections (6 μm) were cut, air dried overnight at room temperature, fixed in acetone for 15 min at −20°C and stored at −70°C before immunostaining.

Immunohistochemical staining

The following monoclonal antibodies were used: anti-Ki67 (Dakopatts, Glostrup, Denmark), anti-EGFR (2E9, Sera Lab Limited, Crawley Down, Sussex, U.K.), anti-cytokeratins number 10, 17, 18 (MNF116, Dakopatts), anti-epithelial glycoprotein (BerEp4, Dakopatts) and anti-LMP1 (CS1-4, Dakopatts). The sections were incubated with the primary antibodies for 30 min at room temperature in a moist chamber. For the LMP1 staining, slides were incubated with primary antibody overnight at 4°C. Interrupted by washings with Tris-buffered saline (TBS), the slides were then incubated with rabbit anti-mouse and swine anti-rabbit immunoglobulin (both peroxidase conjugated, Dakopatts) for 30 min in sequence. The peroxidase reaction was developed with a substrate solution containing H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, Missouri, U.S.A.) for 5 min. The sections were weakly counter-stained with haemotoxylin. In negative controls the primary antibody was replaced by TBS.

Western blot analysis

Frozen tissues were cut into small pieces and transferred to a small volume of RIPA buffer (150 mmol/l NaCl, 50 mmol/l Tris–HCl pH 7.5, 5 mmol/l EDTA, 0.5% sodium deoxycholate, 0.5% sulphonyfluoride), homogenised in a tight-fitting conical glass homogeniser, and sonicated (2 × 15 s) on ice. The lysate was boiled for 5 min and applied to a 7.5% SDS-polyacrylamide gel. After separation, the proteins were transferred to a nitrocellulose filter in a mini-blot apparatus (Bio-Rad, Richmond, California, U.S.A.). The filters were stained with Ponceau S (Sigma) and the position of the molecular weight markers (Bio-Rad) was noted before the filters were destained with water and preabsorbed in 5% non-fat milk in PBS. The filters were then incubated with monoclonal antibody against LMP1, S12 [4], overnight at 4°C and then incubated with antibodies conjugated to IgG alkaline phosphatase antibodies (Bio-Rad) for 2 h. After six washes in 0.5% Tween 20, phosphatase substrate was added.

Measurement of staining results

Serial sections from each specimen were stained with Ki67, EGFR, CS1-4, MNF116 and BerEp4 to distinguish the cancer nest and the stroma component. The Ki67-positive cells were quantified by evaluating randomly selected non-overlapping high-power fields (× 400) representative of tumour area from each section. A high-power field was accepted for evaluation if it did not contain areas of extensive necrosis, normal epithelium or sectioning artifacts. One thousand tumour cells were counted in every case and the proportion of positively stained cells was expressed as percentage. The intensity of EGFR staining for the tumour cells was graded from weak (+/−) to strong (+ + +) based on the intensity of the majority of the tumour cells: +/−, fainter staining than normal nasopharyngeal epithelium; +, same staining as normal nasopharyngeal epithelium; + +, moderate

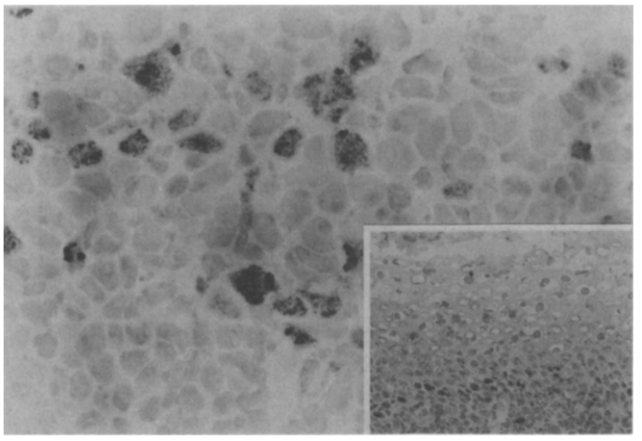

Fig. 1. Immunohistochemical staining for Ki67 in NPC, showing both dot-like and diffuse nuclear staining patterns in tumour cells. Original magnification × 800. Inset: Ki67 is expressed in the nuclei of basal and parabasal cells of stratified squamous epithelium of the nasopharynx. Original magnification × 200.

Table 1. Clinicopathological profiles and expression of Ki67, EGFR and LMP1 in NPC

Case	Sex/age (years)	Histology*	TNM†	Stage‡	LMP1§	EGFR‖	Ki67¶
1	M32	UD	T2N2	III	+	+++	12.7
2	M45	UD	T3N3	IV	+	+++	30.3
3	F61	UD	T2N0	II	+/−	+	2.2
4	M62	UD	T3N3	IV	+	+++	24.2
5	F52	UD	T2N3	IV	++	+++	33.2
6	F39	PD	T3N1	III	+	+	16.2
7	M41	UD	T3N0	III	−	++	4.8
8††	M50	UD	T2N1	II	−	+	1.3
9	M40	UD	—	—	−	+/−	1.2
10	M31	UD	—	—	−	+	1.3
11	F49	UD	T2N0	II	−	+	7.5
12††	M52	PD	T2N0	II	+	++	6.8
13††	F71	PD	T2N0	II	−	+	13.2
14	F57	UD	T2N2	III	−	+/−	3.4
15	F36	PD	T3N1	III	+	++	8.1
16	F37	UD	T2N1	II	−	+	3.2
17	M54	UD	T3N2	III	+	+++	21.2
18	M44	UD	T3N2	III	−	++	2.5
19	M38	UD	T2N2	III	ND	++	15.8
20	M40	UD	T2N0	II	ND	+	8.2
21	M55	UD	T3N2	III	ND	++	22.5
22	M47	UD	T3N2	III	ND	++	17.9
23	M40	UD	T2N3	IV	ND	+	4.3
Control**					−	+	1.9

*Histopathological classification, UD = undifferentiated carcinoma, PD = poorly differentiated squamous cell carcinoma.
†TNM classification.
‡Clinical stages of NPC patients as described in Materials and Methods.
§LMP1 expression detected immunohistochemically, − = negative, +/− = weakly positive, + = moderately positive, ++ = strongly positive.
‖The expression of EGFR was divided into four groups as described in Materials and Methods.
¶The expression of Ki67 was expressed as a percentage of positively stained tumour cells.
**Ten samples of adenoids were used as control in detection of LMP1, EGFR and Ki67 expression in epithelial cells.
††Swedish NPC patients.
ND = not done.

stronger staining than normal nasopharyngeal epithelium; +++, markedly stronger staining than normal nasopharyngeal epithelium.

Statistical analysis

Relationship between the percentage of Ki67-positive cells, EGFR expression and clinical features was evaluated using the Wilcoxon test and χ^2 test. The statistical analyses were carried out with computer software for statistical visualisation, JMP program (SAS Institute Inc. Cary, North Carolina, U.S.A.).

RESULTS

Ki67 staining in normal nasopharyngeal epithelia and NPC

Nuclear immunostaining for Ki67 was seen in cells of the basal and parabasal layer of normal stratified squamous nasopharyngeal epithelium (Fig. 1, inset), and basal cells of respiratory nasopharyngeal epithelium. The Ki67-labelled tumour cells showed both dot-like and a diffuse nuclear staining pattern (Fig. 1). The positive fraction varied from 0.1 to 7.1% in normal nasopharyngeal epithelia (mean 1.9%, S.D. 2.4). As shown in Table 1, the proportion of Ki67-positive tumour cells in NPC ranged from 1.2 to 33.2% (mean 11.4%, S.D. 9.7). The difference between normal nasopharyngeal epithelia and NPC was statistically significant ($P < 0.001$). No significant difference was seen between male and female NPC patients nor between patients under 40 years and over 40 years of age. The correlations between clinical feature and Ki67 expression are summarised in Table 2.

EGFR staining in normal nasopharyngeal epithelia and NPC

Immunostaining for EGFR was seen in the basal cells of respiratory nasopharyngeal epithelium (Fig. 2, inset) and in the basal and parabasal cells of the stratified squamous nasopharyngeal epithelia. EGFR expression was seen in all of the NPC biopsies analysed. EGFR staining showed a predominant cell membrane-associated staining pattern of the tumour cells but with variable intensity while the infiltrating lymphocytes and stroma components showed no reactivity (Fig. 2). In some cases, cancer nests were diffusely stained with EGFR while in other cases tumour cells with variable intensities of EGFR staining were seen. The expression of EGFR in each case is shown in Table 1. As illustrated in Table 2, EGFR expression was related to the tumour stage of NPC. The difference in EGFR expression between negative and positive lymph node metastatic patients was not significant. By combining lymph node negative (N0) and grade 1 lymph node metastatic (N1) patients into one group (both N0 and N1 patients have previously been shown to have a better prognosis [21]), and grade 2 and 3 lymph node metastatic (N2, N3) patients into another group, a statistically significant difference was obtained between these two groups. EGFR expression was not correlated with sex and age of patients.

Table 2. Correlation between clinical features and expression of EGFR and Ki67 antigen in NPC

Clinical feature	No. of cases	EGFR expression*		P†	Ki67 expression‡ (% of positive cell)	P§
		Weak	Strong			
Age < 40 years	9	4	5	NS‖	7.9 (5.9)	NS
Age > 40 years	14	5	9		13.6 (11.1)	
Male	15	5	10	NS	11.7 (10.3)	NS
Female	8	4	4		10.9 (9.7)	
N−¶	6	4	2	NS	7.1 (3.7)	NS
N+	15	5	10		14.5 (10.5)	
N0−1**	10	7	3	<0.05	7.2 (4.7)	<0.05
N2−3	11	2	9		17.1 (10.6)	
T2††	12	8	4	<0.05	9.3 (8.6)	NS
T3	9	1	8		16.4 (9.4)	
II‡‡	7	6	1	<0.01	6.1 (4.2)	<0.05
III+IV	14	3	11		15.5 (10.1)	

*The groups +/− and + were combined and designated as weak expression of EGFR, groups + + and + + + were combined and designated as strong expression of EGFR in this table.
†The significance test was the Fischer's exact χ^2 test.
‡The mean of Ki67-positive cells with standard deviation in each group.
§Data were evaluated by Wilcoxon test.
‖NS = no significant difference.
¶N−, patients with negative lymph nodes; N+, patients with grade 1 to 3 lymph nodes metastasis.
**Patients with negative lymph node and grade 1 positive lymph node are combined to one group. Patients with grade 2 and 3 positive lymph node are combined to another group.
††T stage of tumour according to the TNM classification.
‡‡Clinical stage as described in Materials and Methods.

Expression of LMP1 in NPC

In 9 out of 18 cases, immunostaining for LMP1 in tumour cells gave a dot-like membrane-associated pattern (Fig. 3a). 7 out of 8 cases, with sufficient material for parallel LMP1 analysis by immunohistochemistry and western blot analysis, showed concordant positive results with the expected LMP1 band in the blot (Fig. 3b). The B95.8 marmoset B cell line immortalised by EBV from a patient with infectious mononeucleosis and Rhek-1 epithelial cells, immortalised with Ad12-SV40 hybrid virus, were included as positive and negative controls, respectively. Only the upper band with

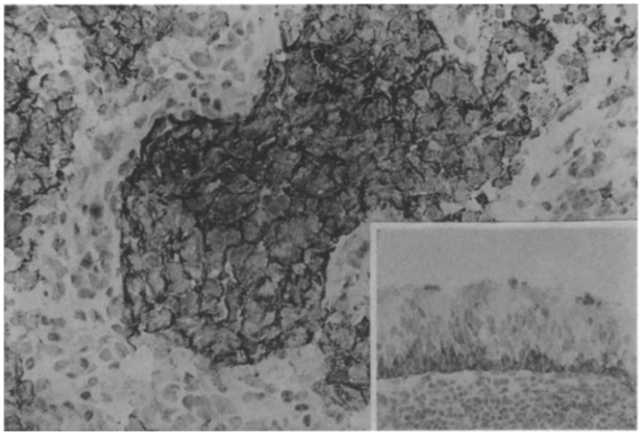

Fig. 2. Immunohistochemical staining showing upregulated EGFR expression in the cancer nest of NPC but not in the stromal component of the tumour. Original magnification × 400. Inset: membrane-associated EGFR expression is seen in the basal cells of respiratory epithelium of the nasopharynx. Original magnification × 200.

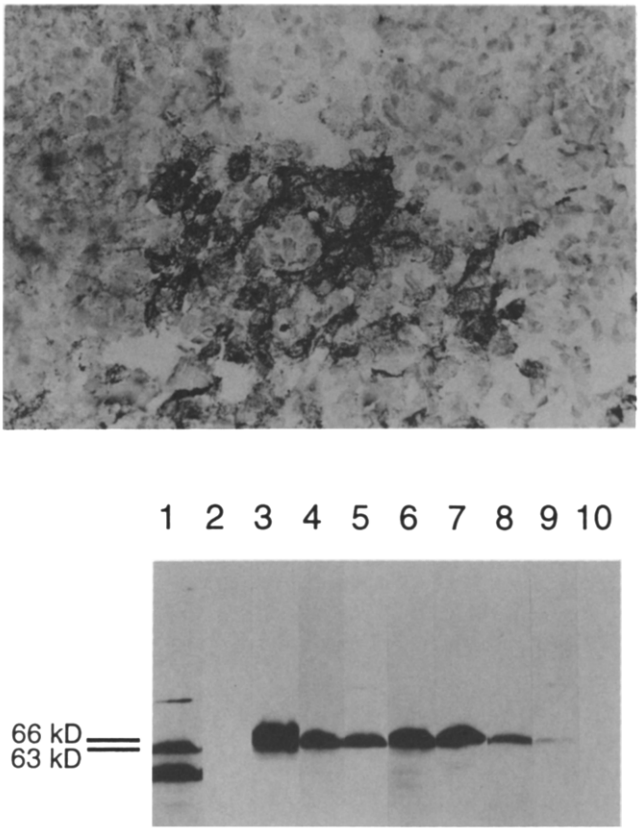

Fig. 3. (a) Immunostaining for LMP1 in NPC shows a dot-like membrane-associated pattern. Original magnification × 400. (b) Western blot of LMP1 expression in NPC. A band at approximately 66 kD in size is seen in 7 out of 8 cases (lanes 3–10). The B95.8 cells (lane 1) and Rhek-1 cells (lane 2) were included as positive and negative controls, respectively.

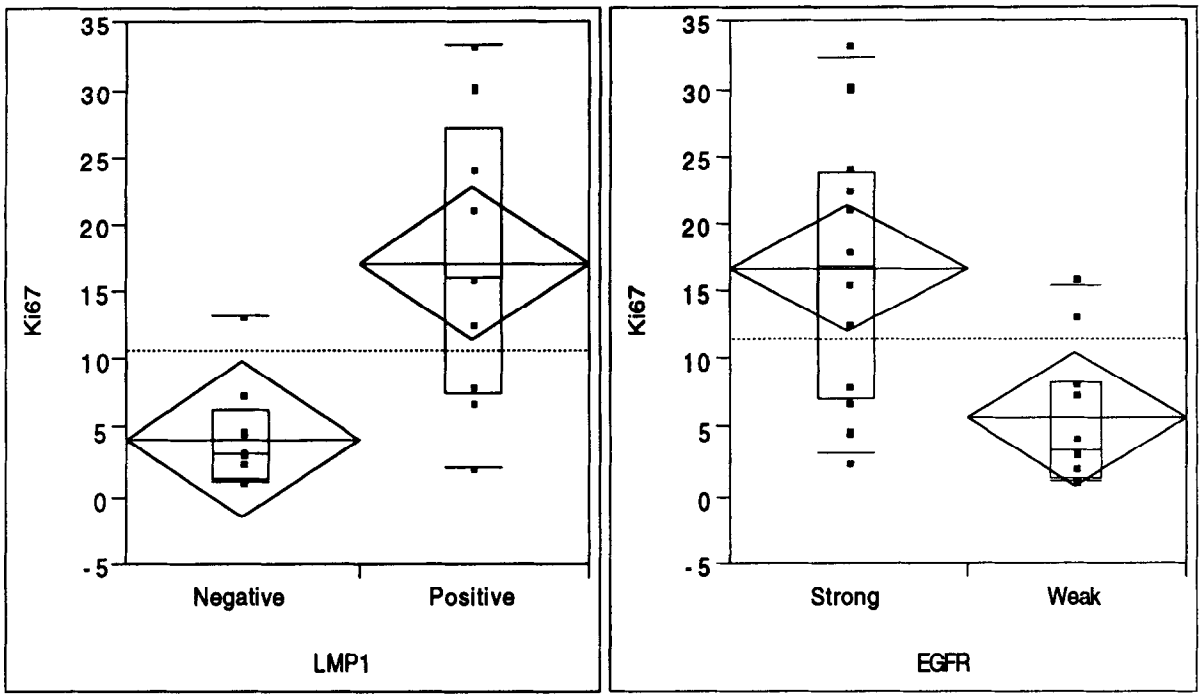

Fig. 4. Expression of Ki67 in comparison with LMP1 and EGFR expression in NPC. The difference for Ki67-positive proportions between the LMP1-positive and -negative groups, as well as strong and weak EGFR expression groups is statistically significant ($P<0.01$).

approximately 63 kD in size in the B95.8 samples was used as standard of B95.8-LMP1. The lower band may be a smaller form of LMP1, present during the lytic cycle of the vius in B95.8 cells. The LMP1 in NPC was slightly larger than B95.8-LMP1 corresponding to the predicted size of the NPC-associated form of LMP1[9]. 4 out of 7 LMP1-negative cases were of stage II NPC while only 2 out of 8 LMP1-positive cases with the same stage. No significant difference was obtained in LMP1 expression related to the sex and age of the patients.

Correlation of expression among Ki67, EGFR and LMP1

As illustrated in Fig. 4, Ki67-positive tumour cells were significantly more frequent in LMP1-positive cases than in LMP1-negative cases (Wilcoxon test, $P<0.01$). Strong expression of EGFR was significantly correlated to a high frequency of Ki67-positive tumour cells in NPC ($P<0.01$). 7 of the 9 LMP1-positive cases showed strong EGFR expression and a high frequency of Ki67-positive tumour cells. In contrast, 7 of the 9 LMP1-negative cases showed weak expression of EGFR and all 9 cases also had lower frequency of Ki67-positive tumour cells.

DISCUSSION

In the present study we found that increased expression of two different growth-associated markers (EGFR and Ki67 antigen) was correlated to the expression of EBV LMP1 in NPC tumour cells. In many tumour types, the tumour cell proliferation rate is related to the clinical course. EGF and the EGFR system have been implicated in the growth regulation of human neoplasms and have also been correlated to prognosis [22]. EBV LMP1 is suggested to contribute to the tumorigenesis of NPC [10]. However, the clinical importance of LMP1 expression in NPC has previously not been addressed. Therefore, an *in situ* study that correlates the expression of LMP1 to cell proliferation-associated antigens, and clinical features is highly relevant.

Ki67, a nuclear antigen expressed in the G1, S, G2 or M phases of the cell cycle, was previously found to inversely correlate with prognosis in non-Hodgkin's lymphomas, sarcomas and carcinomas [12, 23]. In NPC the proportion of Ki67-positive cells correlated both to tumour stage and to EGFR expression suggesting that Ki67 expression might be a prognostic indicator also in NPC.

In normal nasopharyngeal epithelium, the EGFR is expressed exclusively in basal cells coinciding with the histological localisation of Ki67-positive cycling cells. It may indicate that the EGFR signalling is required for epithelial cells to retain a proliferative potential. The increased expression of EGFR in the tumour cells of NPC was also paralleled by a higher frequency of Ki67-positive cells. These findings, as well as the over expression of EGFR reported in other tumours suggest that still unknown mechanism(s) causing unregulated or abnormal EGFR expression may lead to the uncontrolled tumour proliferation [24].

The expression of LMP1 in NPC has previously been detected by western blot analysis [4] and by immunohistochemical staining [25, 26]. We found immunohistochemically detectable LMP1 in 9 out of 18 cases (50%). This is slightly lower than the previously reported 65% found by western blot analysis [4]. The possibility that the immunohistochemical detection of LMP1 is less sensitive than western blot analysis was also indicated by the one discordant case among the 8 cases in parallel analysed by western blot and immunohistochemical analysis. In the present study, EBV DNA was detected in LMP1-negative cases (data not shown). Therefore, negative LMP1 expression is not due to the absence of EBV. We have

previously shown that transfection of Cao-LMP1 in human epithelial cells increases the colony-forming efficiency and the expression of proliferation cell-associated antigen (PCNA) but decreases serum dependency *in vitro* [10]. The findings that NPC cases with positive LMP1 expression showed stronger EGFR expression and a higher frequency of Ki67-expressing cells than the LMP1-negative cases suggest that the expression of LMP1 *in vivo* is also associated with increased proliferation and possibly a more malignant clinical course. A possibility that may be tested is that LMP1 increases tumour cell proliferation via an upregulation of EGFR expression.

Upregulated EGFR expression characterises steroid receptor-negative and lymph node metastatic breast cancer [27], and bladder tumours with poor prognosis as well as late tumour stage in other carcinomas [15]. The over expression of EGFR seems to be an important factor for increasing tumour cell proliferation in a number of human carcinomas. Determination of either expression of Ki67 or EGFR could be used as a marker of tumour growth and poor prognosis. Our findings of a correlation of EGFR expression to the fraction of Ki67-positive tumour cells and to tumour stage in NPC supports the contention that these markers can also be used as predictors of prognosis in NPC.

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