Intermittent abortive reactivation of Epstein-Barr virus during the progression of nasopharyngeal cancer as indicated by elevated antibody levels

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ABSTRACT

The development of nasopharyngeal carcinoma (NPC), a common cancer in Southeastern Asia, is closely associated with Epstein-Barr virus (EBV) infection; however, the aetiological role of EBV in NPC pathogenesis remains enigmatic. The life cycle of EBV in NPC patients is defined as latency II, while the antibodies specific to lytic phase proteins, as well as lytic genes, were highly expressed in NPC patients. The correlation between antibody levels and the progression of NPC has been reported in some studies; however, most of these studies focused on IgA antibodies, and the results in different articles were not consistent. In this study, we concurrently determined the levels of IgA and IgG antibodies specific to six purified recombinant EBV antigens associated with different replication statuses of EBV: EBNA1 associated with latency II; the non-structural antigens Zta, TK, EA-D and EA-R associated with immediate-early and early lytic phases; and the EBV matrix protein VCA p18, which is involved in late lytic phase. Levels of antibodies specific to immediate-early and early antigens were correlated with the tumour progression, especially tumour size. The levels of antibodies specific to some lytic phase antigens were also correlated with lymph node inclusion and metastasis. However, the antibody specific to the latency II antigen EBNA1 was not correlated with either tumour size or metastasis. Consistent with previous transcriptome studies, the results suggested both the expression of lytic phase genes at the protein level and the intermittent reactivation of EBV in NPC patients.

Introduction

Nasopharyngeal carcinoma (NPC) is a common cancer in Southeastern Asia and Africa but is rare in other parts of the world [1]. The development of NPC has been closely associated with Epstein-Barr virus (EBV) infection [2], environmental factors and genetic susceptibility [3]. In 1976, Werner Henle and colleagues reported that serum levels of EBV antibodies of immunoglobulin subtype A (IgA) were generally elevated among nasopharyngeal carcinoma (NPC) patients [4]. Later, a broad spectrum of anti-EBV antibodies, especially immunoglobulin A (IgA), was found to be higher in NPC patients than in healthy carriers and patients with other head and neck diseases [5–14].

In most areas of the world, EBV infection is ubiquitous and lifelong, while only a small fraction of the EBV-infected population develops NPC [15]. Despite the close association of EBV infection with NPC, the etiological role of EBV in NPC pathogenesis remains enigmatic. EBV establishes a latent infection after a pre-latent, short, abortive lytic cycle [16]. There are three types of latency (latency I, latency II and latency III), and the life cycle of EBV in NPC is defined as latency II and is represented by expression of EBNA1, LMP1 and non-coding EBER and BART RNAs [17]. It is generally believed that the virus is involved at early stages of tumour development because the viral genome is regularly present in the intraepithelial lesions [18,19], and it has been reported that the antibody response to tumour-associated virus precedes diagnosis by approximately 3 years [20]. In recent years, it has been found that not only latent genes but also lytic genes, such as

BARF1, BMFL1 and BZLF1, were more highly expressed in NPC [21], suggesting that the reactivation of EBV may occur during the development and progression of NPC.

The correlation between antibody levels and the progress of NPC has been reported in some studies, but the results were not systematic, and some of them were controversial [22–26]. In one study, EBNA1/IgA was found to be correlated with the stage of NPC [25], while in other studies, no correlation was found [23,24]. For other antibodies, such as VCA/IgA, similar problems were also observed [23,26]. In addition to the correlation with NPC stages, controversial results were also found in the correlation between the antibody levels and T or N stage [24,26]. One reason for the controversy was the small sample size. In 4 of the 5 studies, only 20–30 stage I and II NPC cases were included [22–25]. In another study, although the sample size was large enough, only VCA/IgA and EA/IgA were detected and analysed [26]. Another reason is that analysing the data with different statistical methods may also give different conclusions [22,26]. Furthermore, most of these studies focused on IgA, although it has been reported that IgA and IgG responses to EBV antigens were triggered differently [12,14]. However, in that study [12], the antibody profiles were analysed by immunoblotting, during which the proteins were denatured, and the protein levels varied so that it was difficult to compare the reactivity of the proteins to samples representing different stages.

In previous studies, we found that the levels of antibodies specific to immediate-early and early lytic phase antigens were significantly lower in serologically defined high-risk populations compared to NPC patients, and using two-step screening, the positive prediction value could be increased from 4.69% to 18.52% [27]. In the present study, levels of both IgA and IgG antibodies specific to latency II (EBNA1), immediate-early and early EBV-antigen complexes (Zta, TK, EA-D), and the late lytic phase antigen VCA p18 were determined by recombinant antigen-based ELISA assays. In accordance with transcriptome studies [21], the levels of antibodies specific to lytic phase antigens were correlated with both the development and the progression of NPC. These results suggest the abortive reactivation of EBV and the expression of lytic-phase proteins during the development and progression of NPC.

Materials and methods

Serum samples

A total of 277 serum samples from NPC patients were collected at the People's Hospital of Zhongshan, China. Disease status was assessed according to tumour bulk (T), regional lymph node involvement (N) and metastasis (M) based on the staging system of 2008 [28]. A total of 272 samples from NPC patients (including 46 screened NPC samples collected from the screening cohort, which started in 2008 [15]) with TNM staging information were included in this study. All patients were newly diagnosed with histologically confirmed non-keratinizing NPC, and blood was drawn before treatment. A total of 233 serum samples from healthy controls were randomly selected from a screened healthy population. All of the participants were informed, and the study was approved by the Ethical Review Committee of Sun Yat-sen University Cancer Center. All of the sera were stored at $-20\,°C$ for further use.

Protein expression and purification

To determine the levels of antibodies levels specific to proteins representing different replication phases of EBV, six proteins were expressed and purified. The proteins representing the immediate-early and early lytic phases, including Zta, TK, EA-D and EA-R, were expressed and purified as previously reported [29]. For expression of the antigens representing latency II (EBNA1) and the late lytic phase (VCA p18), the gene encoding aa394-641 of EBNA1 and full-length VCA-p18 were synthesized by Sangon Biotech (Shanghai, China). The genes were inserted into PGEX-2T (GE Healthcare, Buckinghamshire, England), and

the expression plasmids carrying the EBV genes were transformed to *Escherichia coli* ER2566 (NEB, Ipswich, MA) for expression. The bacteria were cultured in LB medium at $37\,°C$ until the OD_{600} reached 0.6. IPTG was added to a concentration of 0.8 mM to induce expression, and the bacteria were cultured at $25\,°C$ for another 5 h. The bacteria were collected and lysed by ultra-sonication, and the supernatant containing recombinant EBV antigens was purified by glutathione affinity chromatography. The purity of recombinant EBV peptides was validated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined using the BCA method, and proteins were stored at $-20\,°C$ for further use.

Quantitative detection of anti-EBV IgA and IgG antibodies

The levels of antibodies specific to the immediate-early and early lytic phase antigens Zta, TK, EA-D and EA-R were determined as described in previous studies, and the levels of antibodies specific to EBNA1 and VCA p18 were determined similarly [29]. In brief, 96-well microplates were coated with the purified recombinant proteins (25 ng/well) and blocked with 3% bovine serum albumin in 20 mM PBS. The serum samples were diluted 1:10 with dilution buffer, and 100 μL of diluted samples was added to the pre-coated microplates, followed by incubation at $37\,°C$ for 30 min. After washing, the properly diluted horseradish peroxidase conjugated secondary antibody specific to human IgA and IgG (KPL, Gaithersburg, MD) were added to the microplates to detect IgA and IgG, respectively. After colour development and termination, the optical density (OD) at a wavelength of 450 nm with a reference filter of 620 nm was determined using a Universal Microplate Reader (Bio-Tek, Winooski, VT). The cut-off values were determined as reported in our previous studies [29].

For each plate, a 1.5-fold serially diluted reference serum sample was detected in duplicate, and the standard curves were plotted. The antibody titers of the serum samples were calculated from the corresponding standard curves as described previously [29].

Statistical analysis

Data analysis was performed using SPSS version 18 (Armonk, NY, IBM Corp.) and MedCalc 15.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2015). Pearson's chi-square test was used to analyse the differences in categorical variables. A *t* test was used to analyse the differences in the antibody titers between NPC patients and healthy controls. Receiver operating characteristic analysis was used to analyse the performance of the assays in distinguishing NPC cases from those without NPC using MedCalc 15.2.2. Pearson correlation, linear regression and the Kruskal-Wallis test were performed to examine the relationships between anti-EBV titers and the progression of NPC (Stage and T, N, M). Statistical significance was set at $P < 0.05$.

Results

Serum anti-EBV antibody levels in NPC patients and healthy controls

In the present cross-sectional study, levels of serum anti-EBV antibodies, including IgA and IgG, from 272 NPC patients (including 46 screened NPC cases) and 233 healthy controls were quantitatively determined by an ELISA assay. The median age was 50 (ranging from 29 to 79) and 49 (ranging from 18 to 81) for the healthy controls and NPC patients, respectively, and there was no significant difference between healthy controls and NPC patients regarding sex ($\chi^2 = 0.834$, $P = 0.361$) and age ($F = 0.755$, $P = 0.385$), as analysed by Pearson chi-Square test and one-Way ANOVA (Table 1).

The serum prevalence of EBV was high in Zhongshan, China, as 95.28% of the healthy controls were anti-EBNA1 IgG-positive, and the anti-VCA IgA and IgG serum-positive rates were also above 80%

Table 1
Characteristics of the study population.

Group	Total	Male	Female	Pearson chi-square test	Median age (range)	One-way ANOVA
Healthy Controls	233	161	72	$\chi^2 = 0.834$	50 (29, 79)	F = 0.755
NPC cases	272	198	74	P = 0.361	49 (18, 81)	P = 0.385
Screened NPC[a]	46	30	16	$\chi^2 = 1.605$	52 (30, 68)	F = 1.357
Clinical NPC	226	168	58	P = 0.205	48 (18, 81)	P = 0.245
Stage I	24	22	2	$\chi^2 = 5.939$	49 (34, 71)	F = 0.577
Stage II	75	50	25	P = 0.115	49 (25, 70)	P = 0.630
Stage III	102	73	29		49 (25, 72)	
Stage IV	71	53	18		47 (18, 81)	
T1	64	43	21	$\chi^2 = 4.586$	49 (25, 71)	F = 1.590
T2	44	37	7	P = 0.205	49 (18, 70)	P = 0.192
T3	121	85	36		47 (25, 72)	
T4	43	33	10		50 (31, 81)	
N0	47	37	10	$\chi^2 = 7.274$	48 (29, 71)	F = 1.205
N1	92	60	32	P = 0.064	49 (25, 81)	P = 0.308
N2	103	82	21		49 (25, 72)	
N3	30	19	11		47 (18, 76)	
M0	258	187	71	$\chi^2 = 0.249$	49 (18, 81)	F = 0.469
M1	14	11	3	P = 0.618	47 (28, 67)	P = 0.494

[a] NPC patients who were identified in a screening program using the combination of EBNA1/IgA and VCA/IgA and whose status was confirmed by endoscopy.

(Table 2). The positive rate was lower for antibodies specific to the immediate-early and early lytic phase EBV antigens (Table 2).

The correlation between anti-EBV antibody levels and the development of NPC

Consistent with previous studies, serum antibody levels were higher in NPC patients than in healthy controls, and IgA was better than IgG at distinguishing NPC from non-NPC controls (Fig. 1 and Table 2) [4]. Among the 12 antibody markers detected, EBNA1/IgA, TK/IgA and EA-D/IgA could distinguish NPC from non-NPC well within the areas under ROC curves above 0.90 (Table 2). For most of the EBV antigens, such as EBNA1, Zta, EA-D and VCA p18, there were no significant differences in antibody levels between screened and clinical NPC patients (Fig. 2 and Table 3). However, for TK and EA-R, either IgA or IgG antibody levels were higher in symptomatic NPC patients than fin screened NPC patients (Fig. 2 and Table 3).

The correlation between anti-EBV antibody levels and the stages of NPC

NPC was classified as stage I to IV according to the Chinese 2008 TNM staging system, and there was no significant difference in sex and age among different stage groups as analysed by Pearson's chi-Square

test and one-way ANOVA, respectively (Table 1). The antibody levels of NPC patients diagnosed at different stages are shown in Fig. 2. The correlation between anti-EBV antibody levels and stages of NPC was analysed by a Pearson correlation test, and levels of most antibodies specific to the six selected EBV antigens representing different replication statuses of EBV increased with the progression of NPC (Fig. 2 and Table S1). IgA antibodies specific to the EBNA1 antigen expressed in latency II and IgG antibodies specific to the late lytic phase antigen VCA p18 did not significantly change with the progression of NPC, except anti-EBNA1 IgG (Fig. 2 and Table S1). This result is highly consistent with the results of linear regression analysis (Table S1). In addition, antibodies specific to immediate-early and early lytic phase antigens were significantly higher in stage III and IV tumours than in stage I and II tumours (Table S1). This result may explain why levels of antibodies specific to these antigens were higher in clinical NPC cases than in those identified in screening, as most of the screened NPC cases were identified in the early stage (Table S2).

The correlation between anti-EBV antibody levels and TNM stages

The stages of NPC were dependent on tumour bulk (T), lymph node involvement (N) and metastasis (M), and the T, N and M stages were not independent. Thus, to further analyse the relationship between anti-
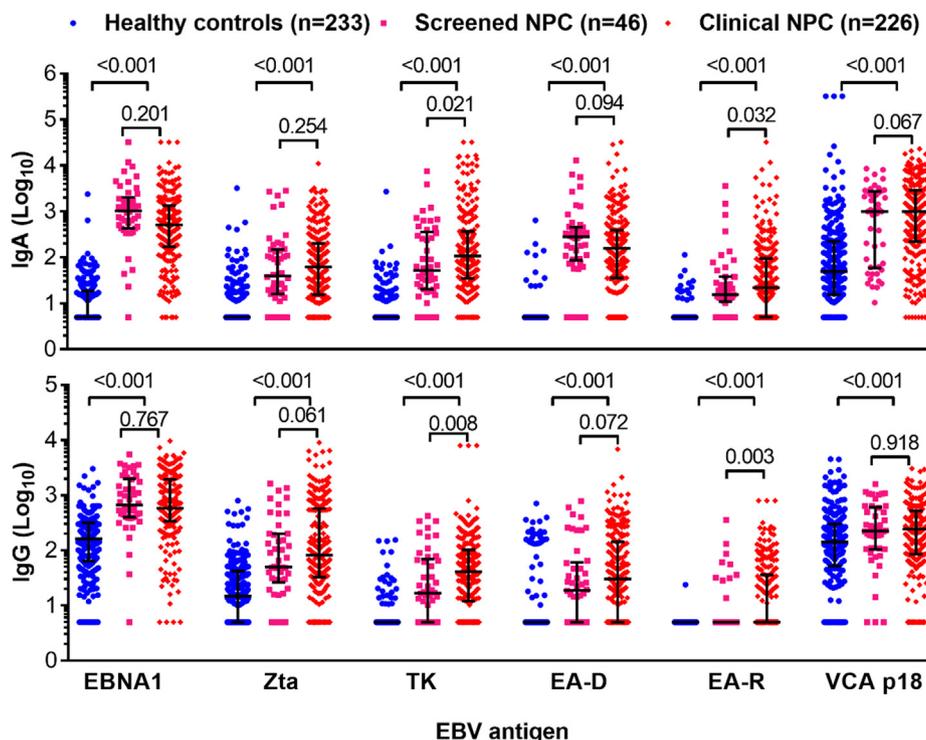
Table 2
The differences in anti-EBV antibody levels in NPC cases and healthy controls.

Antibody	NPC cases (n = 272)		Healthy controls (n = 233)		AUC (95% CI)[a]	Significance
	Antibody titer (GMT)	No. of positive[b] (%)	Antibody titer (GMT)	No. of positive[b] (%)		
EBNA1/IgA	1:453	265 (97.43%)	1:11	116 (49.79%)	0.954 (0.936–0.972)	p < 0.001
Zta/IgA	1:63	162 (59.56%)	1:8	56 (24.03%)	0.865 (0.832–0.898)	p < 0.001
TK/IgA	1:131	251 (92.28%)	1:7	53 (22.75%)	0.932 (0.910–0.955)	p < 0.001
EAD/IgA	1:133	229 (84.19%)	1:6	11 (4.72%)	0.907 (0.879–0.935)	p < 0.001
EAR/IgA	1:33	199 (73.16%)	1:6	16 (6.87%)	0.843 (0.808–0.878)	p < 0.001
VCA/IgA	1:624	265 (97.43%)	1:72	195 (83.69%)	0.781 (0.740–0.821)	p < 0.001
EBNA1/IgG	1:588	267 (98.16%)	1:133	222 (95.28%)	0.814 (0.776–0.852)	p < 0.001
Zta/IgG	1:99	238 (87.50%)	1:30	140 (60.09%)	0.782 (0.741–0.822)	p < 0.001
TK/IgG	1:35	203 (74.63%)	1:6	26 (11.16%)	0.846 (0.811–0.881)	p < 0.001
EAD/IgG	1:34	186 (68.38%)	1:8	26 (11.16%)	0.755 (0.722–0.798)	p < 0.001
EAR/IgG	1:12	98 (36.03%)	1:5	37 (15.88%)	0.681 (0.635–0.726)	p < 0.001
VCA/IgG	1:185	250 (91.91%)	1:112	199 (85.41%)	0.607 (0.558–0.606)	p < 0.001

[a] The area under the ROC as analysed by MedCalc.
[b] The cases were defined as positive with antibody titer ≥ 1:10.

Fig. 1. The elevation in anti-EBV antibody titers in NPC cases compared to healthy controls. The median and interquartile ranges are shown, and the P values are indicated above the scatter dots. The numbers of NPC cases and healthy controls were 272 and 233, respectively.

EBV antibody levels and TNM stages, multivariate linear regression analysis was performed. The results in Table 3 show that most of the changes in the antibody levels were associated with the T stage, except for VCA/IgA and EBNA1/IgA. In addition, TK/IgA, VCA/IgA and TK/IgG were correlated with the N stage, and TK/IgA and EAD/IgG negatively correlated with metastasis of NPC (Table 3). Interestingly, the antibody level of EBNA1/IgG significantly increased as the NPC stage progressed; however, it was not correlated with any of the T, N and M stages (Table 3).

Discussion

In this cross-sectional study, levels of IgG and IgA antibodies specific to six selected EBV antigens representing different EBV life cycles in
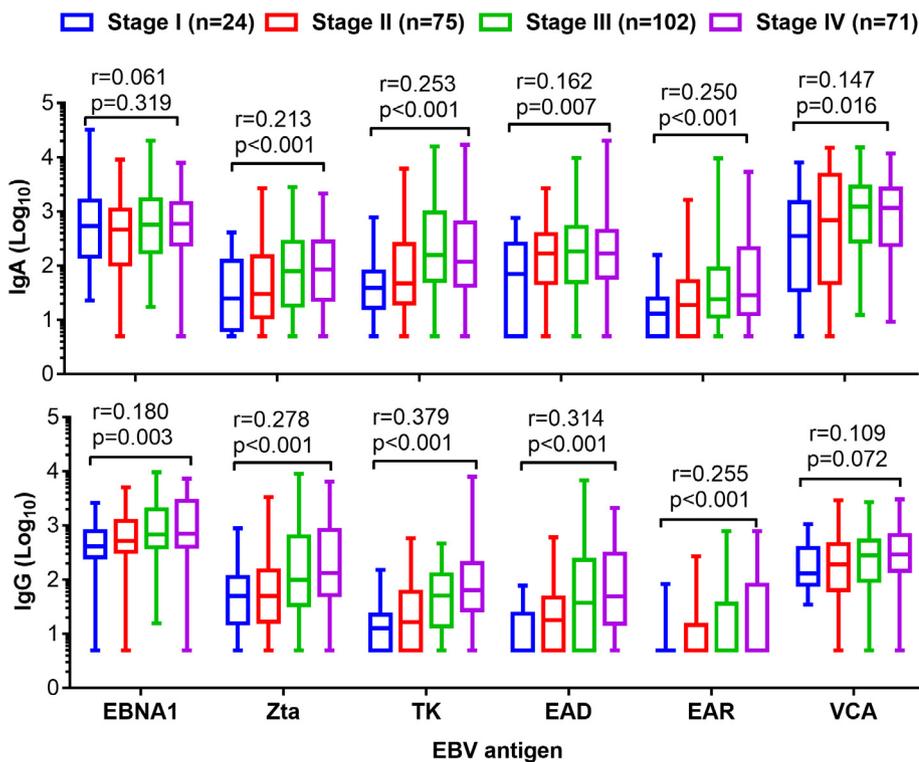

Fig. 2. Correlation between anti-EBV antibody levels and the progression of NPC. The median, interquartile ranges and minimum to maximum values are shown, and the correlation coefficients and P values are given above the boxes and whiskers. The numbers of stage I, II, III and IV NPC cases were 24, 75, 102 and 71, respectively.

Table 3

Correlation between anti-EBV antibody levels and the progression of NPC related to tumour size, lymph node inclusion and metastasis.[a]

Antibody	Stage		T		N		M	
	r	p	r	p	r	p	r	p
EBNA1/IgA	0.060	0.324	–	–	–	–	–	–
Zta/IgA	0.211	< 0.001	0.172	0.005	0.080	0.192	− 0.109	0.074
TK/IgA	0.264	< 0.001	0.203	0.001	0.122	0.047	− 0.128	0.037
EA-D/IgA	0.163	0.007	0.173	0.005	0.018	0.768	− 0.062	0.309
EA-R/IgA	0.253	< 0.001	0.233	< 0.001	0.009	0.882	− 0.066	0.283
VCA/IgA	0.147	0.016	0.037	0.543	0.134	0.029	< 0.001	0.996
EBNA1/IgG	0.176	0.004	0.113	0.065	0.092	0.133	0.005	0.937
Zta/IgG	0.275	< 0.001	0.180	0.003	0.104	0.088	− 0.024	0.696
TK/IgG	0.378	< 0.001	0.250	< 0.001	0.152	0.013	− 0.090	0.142
EA-D/IgG	0.316	< 0.001	0.251	< 0.001	0.100	0.102	− 0.141	0.021
EA-R/IgG	0.255	< 0.001	0.197	0.001	0.018	0.768	0.010	0.867
VCA/IgG	0.111	0.068	–	–	–	–	–	–

[a] Linear regression was used to analyse the correlation between anti-EBV antibody levels and the progression of NPC related to tumour size, lymph node inclusion and metastasis. During the regression analysis, sex and age were also included as independent factors, and the method "enter" was chosen. "r" indicates the partial coefficient in linear regression.

NPC patients were compared to those in healthy controls, and the levels of these antibodies were related to the development and progression of the cancer. It was found that levels of antibodies specific to immediate-early and early lytic phase antigens were not only correlated with the development of NPC but also with NPC the progression. The results suggested the intermittent reactivation of EBV and the expression of lytic phase proteins in NPC.

In most areas of the world, EBV infection is ubiquitous and lifelong. In China, approximately 50% of children are infected by EBV before one year of age, and the rate increases gradually to approximately 90% before ten years of age [30]. Similar results were found in this study; above 95% of the healthy controls were EBV-seropositive (Table 2). Nonetheless, only a small fraction of the EBV-infected population develops NPC [15]. Consistent with previous studies, anti-EBV antibodies were elevated in NPC patients compared to healthy controls [5–10], and these antibodies, especially EBNA1/IgA, TK/IgA and EA-D/IgA, were useful for NPC screening (Table 2). As previously reported, the elevation in IgA antibodies was more vigorous compared to that in IgG antibodies (Table 2) [4,12], and supports the hypothesis of Ho et al. that such outstanding serological features of NPC are largely attributed to mucosal antibody responses to the tumour-associated virus [31].

Regarding the correlation between anti-EBV antibodies and the development of NPC, few studies have focused on the correlation between replication of EBV and the progress of NPC, and most of these results are controversial [22–25]. Small sample sizes and the use of different statistical methods could contribute to the controversial results. In addition, few antigens have been studied for this correlation, and most of these studies focused on IgA, although it has been reported that IgA and IgG responses to EBV antigens are triggered differently [12].

In this study, among the 272 NPC cases, 99 were identified in stage I and II, and both IgA and IgG antibodies specific to the six selected EBV antigens representing latency II and immediate-early, early and late lytic phases of EBV were determined. To our knowledge, this is the first systematic analysis of the correlation between anti-EBV antibody levels and the progression of NPC. It is not surprising that the responses by NPC patients to EBNA1 were more vigorous than those to other antigens (Fig. 1 and Table 2), as only latency II, which is accompanied by regular expression of EBNA1, [17,32], is shown in epithelial cells and detected in undifferentiated NPC [33]. In addition, anti-EBNA1 IgA appeared to peak during early-stage NPC and remained relatively constant as the NPC progressed (Fig. 2 and Table S1). The antibodies specific to lytic antigens were elevated as the NPC progressed (Fig. 2, Table 3 and Table S1). This result is consistent with that of previous studies, which showed that some lytic genes, such as BARF1, BMFL1

and BZLF1, were more highly expressed in NPC [21], suggesting the expression of these lytic genes at the protein level. It is speculated that although EBV is primarily maintained in type II latency in NPC, reactivation may occur.

As analysed by multivariate linear regression, we found that elevated levels of antibodies to the immediate-early and early antigens increased with tumour progression, especially with the increase in tumour bulk (Table 3). The correlation between EA/IgA and the T stage has also been reported in previous studies [26], while two other studies observed no significant correlation [24,25]. In addition to EA/IgA, no correlation between Zta/IgA and the T stage was found in those two studies [24,25]. The small sample size could be the key factor causing the controversy of the different studies, as the conclusion was the same for the antibodies specific to immediate-early and early antigens, regardless of the statistical methods used (Table S3).

The immediate-early gene BZLF1 encodes Zta, which is considered the molecular switch for EBV reactivation and enhances the expression of viral early genes [34]. Lytic replication could enhance the expression of viral and cellular growth factors and cytokines that mediate the infiltration of lymphocytes [35]. While a high level of infiltration is the typical pathological hallmark of NPC, it is considered crucial for the development and maintenance of the tumour [16]. Furthermore, the secretion of viral and cellular growth factors and cytokines could also promote the cell cycle of neighbouring cells, which could be correlated with tumour bulk progression. Thus, intermittent reactivation occurred with increasing frequency as the tumour progressed and with the expression of immediate-early and early genes (Zta, TK, EA-D and EA-R) [17].

Some antibody levels were also correlated with node involvement or metastasis, such as TK/IgA, VCA/IgA, TK/IgG and EA-D/IgG (Table 3). TK/IgA and TK/IgG were correlated with N stage, regardless of the statistical methods used (Table S3). Consistent with the results of Sun et al. VCA/IgA was correlated with the N stage, and productive reactivation may have occurred in the lymph node [26]. However, it was difficult to explain why antibodies specific to Zta, EA-D and EA-R or VCA/IgG were not correlated with N stage (Table 3). It should also be noted that the correlation coefficients for the N and M stages were smaller than that for the T stage (Table 3). It seems likely that the response of patients with node involvement or distant metastasis probably occurs because the disease might affect the response of EBV-harbouring cells not associated with the NPC tumour.

Finally, it should be noted that different statistical analyses could also give different results; more antibodies were correlated with N stage in the Pearson correlation analysis and Kruskal-Wallis test than in linear regression analysis (Table S3). The tumour bulk, lymph node inclusion

and metastasis were not completely independent; the larger the tumour, the higher the probability of lymph node and distal metastasis. Thus, multivariate analysis or linear regression may give more reliable statistical results than the tests mentioned above.

In summary, levels of antibodies specific to EBV antigens that represent different EBV life cycles were analysed, and the elevation of antibodies specific to immediate-early and early lytic antigens was correlated with tumour bulk. These results suggested the expression of lytic genes at the protein level and the occurrence of intermittent lytic replication in NPC patients. However, only the role of BZLF1 (Zta) has been described in previous studies [16], and the potential roles of these antigens in the development and progress of NPC remain unclear and should be verified in future studies.

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Conflict of interest

There was no conflict of interest for all of the authors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.04.024.

References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65:87–108.

[2] Tang LL, Chen WQ, Xue WQ, He YQ, Zheng RS, Zeng YX, et al. Global trends in incidence and mortality of nasopharyngeal carcinoma. Cancer Lett 2016;374:22–30.

[3] Tsao SW, Yip YL, Tsang CM, Pang PS, Lau VM, Zhang G, et al. Etiological factors of nasopharyngeal carcinoma. Oral Oncol. 2014;50:330–8.

[4] Henle G, Henle W. Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int J Cancer 1976;17:1–7.

[5] Connolly Y, Littler E, Sun N, Chen X, Huang PC, Stacey SN, et al. Antibodies to Epstein-Barr virus thymidine kinase: a characteristic marker for the serological detection of nasopharyngeal carcinoma. Int J Cancer 2001;91:692–7.

[6] Zhang X, Zhang Y, Nie Y, Wang S, Chen Y, Sun D. Serum Zta antibody of Epstein-Barr virus exerts potential function in the diagnosis of nasopharyngeal cancer. Tumour Biol: J Int Soc Oncodevelop Biol Med 2014;35:6879–86.

[7] Littler E, Newman W, Arrand JR. Immunological response of nasopharyngeal carcinoma patients to the Epstein-Barr-virus-coded thymidine kinase expressed in Escherichia coli. Int J Cancer 1990;45:1028–32.

[8] Gu AD, Lu LX, Xie YB, Chen LZ, Feng QS, Kang T, et al. Clinical values of multiple Epstein-Barr virus (EBV) serological biomarkers detected by xMAP technology. J Transl Med 2009;7:73.

[9] Zhu XX, Zeng Y, Wolf H. Detection of IgG and IgA antibodies to Epstein-Barr virus membrane antigen in sera from patients with nasopharyngeal carcinoma and from normal individuals. Int J Cancer 1986;37:689–91.

[10] Wout MJ, Van Grunsven WJMS, Middeldorp Jaap M. Localization and diagnostic application of immunodominant domains of the BFRF3-encoded epstein-barr virus capsid protein. J Infect Dis 1994;170:13–9.

[11] Coghill AE, Bu W, Hsu WL, Nguyen H, Yu KJ, Chien YC, et al. Evaluation of total and IgA-specific antibody targeting epstein-barr virus glycoprotein 350 and nasopharyngeal carcinoma risk. J Infect Dis 2018;218:886–91.

[12] Fachiroh J, Schouten T, Hariwiyanto B, Paramita DK, Harijadi A, Haryana SM, et al. Molecular diversity of Epstein-Barr virus IgG and IgA antibody responses in nasopharyngeal carcinoma: a comparison of Indonesian, Chinese, and European

[13] Middeldorp JM. Epstein-barr virus-specific humoral immune responses in health and disease. Curr Top Microbiol Immunol 2015;391:289–323.

[14] Chen H, Chen S, Lu J, Wang X, Li J, Li L, et al. Multiparametric detection of antibodies against different EBV antigens to predict risk for nasopharyngeal carcinoma in a high-risk population of China. Cancer Prev Res (Phila) 2017;10:542–50.

[15] Liu Z, Ji MF, Huang QH, Fang F, Liu Q, Jia WH, et al. Two Epstein-Barr virus-related serologic antibody tests in nasopharyngeal carcinoma screening: results from the initial phase of a cluster randomized controlled trial in Southern China. Am J Epidemiol 2013;177:242–50.

[16] Murata T, Tsurumi T. Switching of EBV cycles between latent and lytic states. Rev Med Virol 2014;24:142–53.

[17] Murata T, Sato Y, Kimura H. Modes of infection and oncogenesis by the Epstein-Barr virus. Rev Med Virol. 2014;24:242–53.

[18] Sham JS, Wei WI, Zong YS, Choy D, Guo YQ, Luo Y, et al. Detection of subclinical nasopharyngeal carcinoma by fibreoptic endoscopy and multiple biopsy. Lancet 1990;335:371–4.

[19] Yip TT, Ngan RK, Fong AH, Law SC. Application of circulating plasma/serum EBV DNA in the clinical management of nasopharyngeal carcinoma. Oral Oncol 2014;50:527–38.

[20] Ji MF, Wang DK, Yu YL, Guo YQ, Liang JS, Cheng WM, et al. Sustained elevation of Epstein-Barr virus antibody levels preceding clinical onset of nasopharyngeal carcinoma. Br J Cancer 2007;96:623–30.

[21] Hu L, Lin Z, Wu Y, Dong J, Zhao B, Cheng Y, et al. Comprehensive profiling of EBV gene expression in nasopharyngeal carcinoma through paired-end transcriptome sequencing. Front Med 2016;10:61–75.

[22] Shao JY, Li YH, Gao HY, Wu QL, Cui NJ, Zhang L, et al. Comparison of plasma Epstein-Barr virus (EBV) DNA levels and serum EBV immunoglobulin A/virus capsid antigen antibody titers in patients with nasopharyngeal carcinoma. Cancer 2004;100:1162–70.

[23] Fachiroh J, Paramita DK, Hariwiyanto B, Harijadi A, Dahlia HL, Indrasari SR, et al. Single-assay combination of Epstein-Barr Virus (EBV) EBNA1- and viral capsid antigen-p18-derived synthetic peptides for measuring anti-EBV immunoglobulin G (IgG) and IgA antibody levels in sera from nasopharyngeal carcinoma patients: options for field screening. J Clin Microbiol 2006;44:1459–67.

[24] Cai Y, Zheng YM, Cheng JR, Wang W, Zhang YN, Wang WH, et al. Relationship between clinical stages of nasopharyngeal carcinoma and Epstein-Barr virus antibodies Rta/IgG, EBNA1/IgA, VCA/IgA and EA/IgA. Nan Fang Yi Ke Da Xue Xue Bao 2010;30:509–11.

[25] Cai YL, Li J, Lu AY, Zheng YM, Zhong WM, Wang W, et al. Diagnostic significance of combined detection of Epstein-Barr virus antibodies, VCA/IgA, EA/IgA, Rta/IgG and EBNA1/IgA for nasopharyngeal carcinoma. Asian Pacific J Cancer Prevent: APJCP. 2014;15:2001–6.

[26] Sun Peng, Chen Cui, Cheng Yi-Kan, Zeng Zhi-Jian, Chen Xin-Lin, Liu Li-Zhi, Gu Mo-Fa. Serologic biomarkers of Epstein–Barr virus correlate with TNM classification according to the seventh edition of the UICC/AJCC staging system for nasopharyngeal carcinoma. Eur Arch Otorhinolaryngol 2014;271(9):2545–54. https://doi.org/10.1007/s00405-013-2805-5.

[27] Li T, Guo X, Ji M, Li F, Wang H, Cheng W, et al. Establishment and validation of a two-step screening scheme for improved performance of serological screening of nasopharyngeal carcinoma. Cancer Med 2018;7:1458–67.

[28] Mao YP, Li WF, Chen L, Sun Y, Liu LZ, Tang LL, et al. A clinical verification of the Chinese 2008 staging system for nasopharyngeal carcinoma. Chin J Cancer 2009;28:1022–8.

[29] Min XP, Zhang MZ, Yuan SS, Ge SX, Liu XR, Zeng XX, et al. Using MOEA with redistribution and consensus branches to infer phylogenies. Int J Mol Sci. 2018;19.

[30] Xiong G, Zhang B, Huang MY, Zhou H, Chen LZ, Feng QS, et al. Epstein-Barr virus (EBV) infection in Chinese children: a retrospective study of age-specific prevalence. PloS One 2014;9:e99857.

[31] Ho HC, Ng MH, Kwan HC, Chau JC. Epstein-Barr-virus-specific IgA and IgG serum antibodies in nasopharyngeal carcinoma. Br J Cancer 1976;34:655–60.

[32] Young LS, Arrand JR, Murray PG. EBV gene expression and regulation. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, et al., editors. Human herpesviruses: biology, therapy, and immunoprophylaxis. Cambridge; 2007.

[33] Munz C, Moormann A. Immune escape by Epstein-Barr virus associated malignancies. Semin Cancer Biol 2008;18:381–7.

[34] Speck SH, Chatila T, Flemington E. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. Trends Microbiol 1997;5:399–405.

[35] Hsu M, Wu SY, Chang SS, Su IJ, Tsai CH, Lai SJ, et al. Epstein-Barr virus lytic transactivator Zta enhances chemotactic activity through induction of interleukin-8 in nasopharyngeal carcinoma cells. J Virol 2008;82:3679–88.