Expression and clinical significance of programmed death ligand 1 in nasopharyngeal carcinoma

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Abstract. Nasopharyngeal carcinoma (NPC) has the highest incidence of all types of head and neck cancer in China. The present study aimed to investigate the association between the expression of programmed death ligand 1 (PD-L1) in NPC tissues and clinicopathological features, as well the outcomes for NPC patients. In addition, the association between tissue expression of PD-L1 and immune components in peripheral blood was assessed. The expression of PD-L1 was determined by immunohistochemistry, while immune indexes were determined by ELISA and flow cytometry. The positive expression rate of PD-L1 in NPC patients was 29.2%, and the PD-L1 expression levels were associated with distant metastasis (P=0.010) and the T-stage of the primary tumor (P=0.032). The expression of PD-L1 was associated with the distant metastasis-free survival of NPC patients (P=0.006). In addition, a statistically significant association of PD-L1 expression with Epstein-Barr virus viral capsid antigen IgA (EBV VCA-IgA; P=0.046) and with $CD3^-CD19^+$ cells (P=0.014) was identified. These results indicated that PD-L1 may be a potential prognostic biomarker for NPC patients, and that EBV VCA-IgA and $CD3^-CD19^+$ cells may be useful for predicting PD-L1 expression when its levels cannot be detected due to the lack of a tumor tissue sample.

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Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor type that arises from the epithelial surface of the nasopharynx, and has the highest incidence of all types of head and neck cancer in China. Men are most frequently affected by NPC, typically at an age of 40-50 years. Causative factors for NPC include heredity, Epstein-Barr virus (EBV) and environmental factors (1). Primary NPC lesions are differentiated or undifferentiated non-keratinizing carcinomas, which are sensitive to radiotherapy. As the radiotherapy response rate is 85%, it is the preferred treatment for NPC (2). However, due to the variable clinical manifestations of NPC, misdiagnosis and missed diagnosis occur frequently. The majority of patients are diagnosed in the late stages of the disease, when the tumor has invaded the surrounding tissues; the higher local recurrence rates and distant metastasis rates associated with late-stage NPC lead to decreased overall survival (3). Therefore, a novel and effective method for the clinical diagnosis of NPC is urgently required.

The occurrence of NPC is associated with EBV infection, and immune function serves an important role in the development of NPC. The expression of programmed death ligand 1 (PD-L1) has been reported as a reliable prognostic factor for various types of malignancy. However, the association between the expression of PD-L1 and the clinicopathological factors and prognosis of NPC patients remain to be fully elucidated.

PD-L1 is a member of the B7 family and the protein consists of 290 amino acids (4). The extracellular domain of PD-L1 binds to programmed cell death protein 1 (PD-1), which is expressed on the surface of activated T lymphocytes. The PD-1/PD-L1 pathway mediates negative co-stimulatory signals to effectively inhibit T-cell function and proliferation, and decrease the secretion of cytokines, including interleukin (IL)-2, IL-10 and interferon (IFN)-γ. The pathway serves an important role in the immunomodulation of various diseases and pathological processes, including tumor immunity, transplantation immunity, viral infection and autoimmunity (5,6). The abnormal expression of PD-L1 in numerous types of malignant tumor, including melanoma, lung cancer, esophageal cancer, colorectal cancer, breast cancer and glioma, is associated with tumor invasion and metastasis, decreased tumor infiltration by T lymphocytes, poor prognosis and reduced survival time (7).

NPC is characterized by prevailing EBV infection and the presence of immune cell infiltration around the cancer lesions (8). EBV-DNA exists as cell-free fragments in the serum of infected patients. Lo *et al* (9) identified that EBV-DNA was detectable in the plasma of 96% of the NPC patients assessed, and observed that serum EBV-DNA levels in patients with advanced-stage disease were significantly higher than that in patients with early-stage disease, suggesting that serum EBV-DNA levels may be positively correlated with the tumor burden. EBV early antigen (EA)-immunoglobulin (Ig)A and EBV viral capsid antigen (VCA)-IgA are markers of viral infection that are commonly used in the clinical screening and auxiliary diagnosis of NPC, and indicate the risk of NPC development. It has been identified that NPC patients display aberrations in peripheral blood lymphocyte subsets (10); this abnormal immune status may be due to the abnormal cellular immune response in NPC and may be associated with the immune response to EBV infection. However, the possible underlying association between PD-L1 and alterations in the immune components in the peripheral blood of NPC patients has remained to be determined.

In the present study, the expression of PD-L1 was examined in NPC tissues of patients from northern China, and the association between PD-L1 expression levels and the clinicopathological parameters was assessed. In addition, the lymphocyte subsets, EBV-DNA and EBV antibodies in peripheral blood were assessed in order to provide potential markers for the diagnosis and treatment of NPC.

Materials and methods

Patients and clinical data. Tissue specimens and peripheral blood were collected from 96 NPC patients, including 72 males (median age, 48 years; range, 13-82 years) and 24 females (median age, 41.5 years; range, 11-67 years). Patients were recruited at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). The patients were enrolled according to the following criteria: i) Radiotherapy was received; ii) complete clinical data were available; and iii) no other malignant diseases, active hepatitis or diabetes were diagnosed. The retrospective study was approved by the Institutional Review Board of the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China. The trial registration number was NCC2016YJC-10). Written, informed consent was provided by all patients prior to enrollment in the study.

Immunohistochemical analysis. The paraffin-embedded tissues were cut into 4-μm sections, dewaxed and then rehydrated with a graded ethanol series. To quench endogenous peroxidase activity, the sections were incubated with 3% H_2O_2 at room temperature for 20 min. For antigen retrieval, the sections were heated in ethylene diamine tetraacetic acid buffer (pH, 8.0) for 2 min using a pressure cooker. The slides were blocked with normal goat serum (Ready-to-use; cat. no. ZLI-9022; OriGene Technologies, Beijing, China) at 37˚C for 20 min. Samples were incubated with rabbit-monoclonal anti-PD-L1 antibody (dilution, 1:200; cat. no. ab205921; Abcam, Cambridge, UK) and mouse monoclonal anti-P16 antibody (Ready-to-use; cat. no. 705-4713; Ventana Medical Systems, Inc., Tucson, AZ, USA) overnight at 4˚C. Incubation with secondary antibody and staining was performed using an immunohistochemical staining kit (cat. no. PV-9000; OriGene Technologies) according to the manufacturer's protocol. The immunoreaction was visualized with diaminobenzidine staining, followed by counterstaining with hematoxylin. The primary antibody was replaced with PBS to prepare the negative control sample, and PD-L1-positive slides incubated with the antibodies were used as a positive control.

Percentages of PD-L1-positive tumor cells and staining intensity were independently evaluated by two experienced pathologists who were blinded to the clinical information. They assessed 20 sequential high-power fields (0.54 mm diameter per field) using Aperio ImageScope software version 12.3.0 (Leica Biosystems, Wetzlar, Germany) to quantify the staining in the images captured. A proportion of stained cells of >10% was considered to indicate a positive expression status, with >25% considered as high expression and 10-25% as low expression.

Lymphocyte subset detection. Venous blood samples were collected from patients prior to radiotherapy and stored with K3EDTA anti-coagulant. The samples were analyzed by flow cytometry using the BD Multitest™ IMK kit (cat. no. 340503; BD Biosciences, Franklin Lakes, NJ, USA). The CD4/CD8/CD3/CD45 trichromatic fluorescent monoclonal antibodies in the kit were diluted 1:10 and incubated with the samples for 15 min at room temperature. FACSCanto version 6.1.3 (BD Biosciences) was used to analyze T-lymphocyte subsets, including total T lymphocytes ($CD3^+$T), the T auxiliary/induced cell subset ($CD3^+CD4^+$T), the T suppressor/cytotoxic cell subset ($CD3^+CD8^+$T), the ratio of T auxiliary to suppressor cells ($CD3^+CD4^+/CD3^+CD8^+$), natural killer (NK) cells ($CD3^-CD56^+$) and B lymphocytes ($CD3^-CD19^+$).

Detection of EBV infection. ELISA diagnostic kits (Euroimmun, Lübeck, Germany) were applied to detect serum EBV EA-IgA (cat. no. EI 2795-9601 A) and VCA-IgA (cat. no. EI 2791-9601 A) antibodies. The kits were used according to the manufacturer's protocol, and the antibody concentrations were determined with a standard curve. EBV-DNA was detected using the Epstein-Barr Virus (EBV) Polymerase Chain Reaction (PCR) Fluorescence Detection kit (cat. no. DA-D065; DaAn Gene Co., Ltd., Guangzhou, China) according to the manufacturer's protocol, with a Roche LightCycler® 480II (Roche Diagnostics, Basel, Switzerland).

Follow-up. Following the completion of radiotherapy and chemotherapy, the NPC patients were followed up every 3 months, which included thoracic and abdominal computed tomography scans, and nasopharyngeal and cervical magnetic resonance imaging (MRI). When a nasopharyngeal mass was identified on MRI, electro-epipharyngoscopy and biopsy were performed. Fine-needle aspiration cytology was applied when cervical lymph node enlargement was identified. Follow-up continued until June 1, 2017; the last time-point for the patient with the latest enrolment. Distant metastasis-free survival (DMFS) was defined as the time from the beginning of radiotherapy to the identification of metastasis, or until the

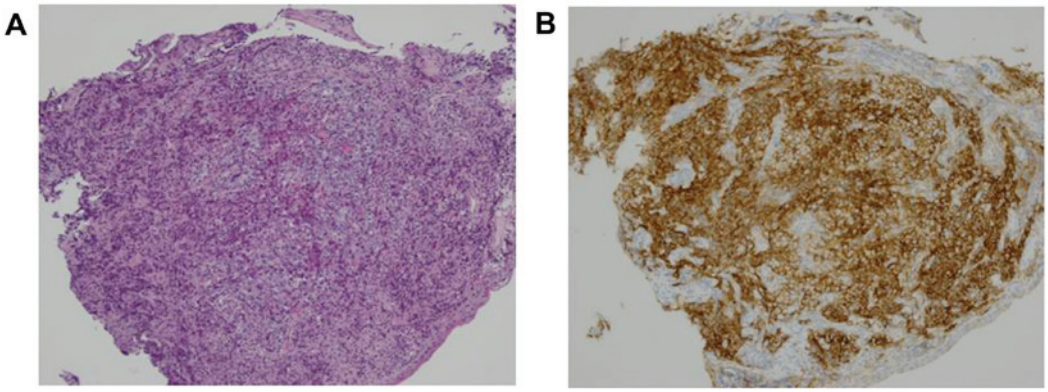

Figure 1. Representative images of H&E staining and immunohistochemical staining for PD-L1 in tumor samples from NPC patients. (A) H&E staining of an NPC tumor sample (magnification, x100). (B) Representative immunohistochemical image of an NPC tumor sample with expression of PD-L1 (magnification, x100). H&E, hematoxylin and eosin; PD-L1, programmed death ligand 1; NPC, nasopharyngeal carcinoma.

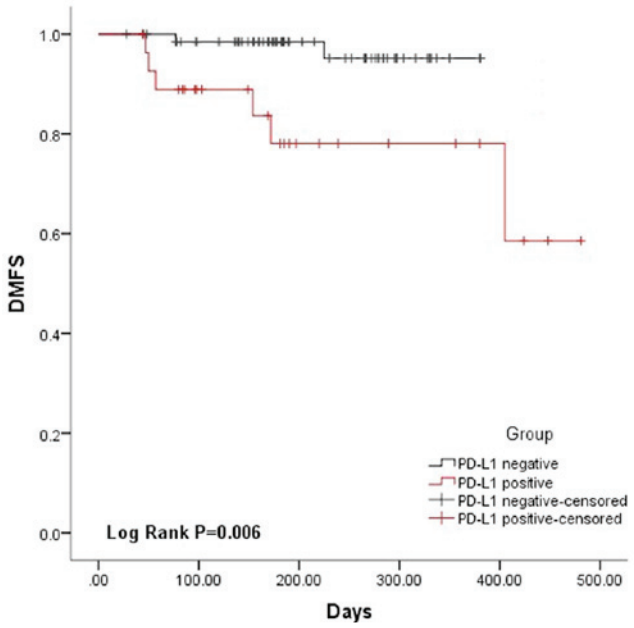

Figure 2. DMFS survival analysis of nasopharyngeal carcinoma patients. Kaplan-Meier survival curves for patient DMFS time stratified by PD-L1 expression levels (χ^2=7.485, P=0.006). DMFS, distant metastasis-free survival; PD-L1, programmed death ligand 1.

last follow-up. The distant metastases were all detected after 1 year's follow-up.

Statistical analysis. SPSS version 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Discrete variables were compared using Fisher's exact test, χ^2 test and Wilcoxon W test, continuous variables were compared using Student's t-test, Mann-Whitney U and Kruskal-Wallis H test. Survival data were analyzed using the Log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of PD-L1 protein in NPC patients. The expression of PD-L1 was analyzed in 96 NPC specimens by immunohistochemical staining. The protein was predominantly expressed on the cell membrane (Fig. 1). Immunohistochemical analysis revealed that 28/96 NPC samples (29.2%) were positive for PD-L1 expression and 68 cases (70.8%) were negative.

PD-L1 expression and the clinicopathological features of NPC patients. The association of PD-L1 expression with clinicopathological parameters was assessed to clarify the function of PD-L1 in NPC. No significant associations between PD-L1 expression status and sex, age or histotype classification were identified. However, PD-L1 expression was significantly associated with NPC 1-year distant metastasis (P=0.010) and the tumor (T)-stage of the primary tumor (P=0.032). No significant association between PD-L1 expression and the AJCC stage (2010) or treatment modality (with or without chemotherapy or before or after radiotherapy) was identified (Table I).

Expression of PD-L1 is associated with the DMFS of NPC patients. The DMFS for the PD-L1-positive and -negative groups is presented in Fig. 2. Patients with a PD-L1-positive status had significantly poorer DMFS (P=0.007) compared with that of patients with a PD-L1-negative status. The 1-year distant metastasis rate was 2.9% for PD-L1-negative patients and 21.4% for PD-L1-positive patients (Table I). Thus, the patients with a PD-L1-positive status were at a significantly higher risk of developing metastasis.

Association of PD-L1 expression status in NPC tissues with EBV-DNA and lymphocyte subsets in the peripheral blood. PD-L1 is an immunosuppressive molecule associated with an abnormal immune status in the body. ELISA and qPCR were used to detect the levels of EBV antibodies and EBV-DNA, while lymphocyte subsets were detected using flow cytometric analysis of the peripheral blood of NPC patients. A statistically significant association of PD-L1 expression with EBV VCA-IgA antibodies (P=0.046) and with $CD3^-CD19^+$ cells was identified (P=0.014; Table II).

Discussion

NPC is associated with EBV infection, poor tumor cell differentiation and sensitivity to chemotherapy. In addition, compared with other types of head and neck squamous cell carcinoma, NPC has a unique geographical distribution, with

Table I. Association between PD-L1 expression and the clinicopathological features of nasopharyngeal carcinoma patients.

Characteristic	Cases (n)	PD-L1-negative (%)	PD-L1-positive (%)	χ^2	P-value
Sex				0.269	0.604
Male	72	52 (72.2)	20 (27.8)		
Female	24	16 (66.7)	8 (33.3)		
Age (years)				1.18	0.277
>46	46	35 (76.1)	11 (23.9)		
≤46	50	33 (66.0)	17 (34.0)		
Histotype				1.803	0.179
Undifferentiated non-keratinizing carcinoma	64	42 (65.6)	22 (34.4)		
Differentiated non-keratinizing carcinoma	27	22 (81.5)	5 (18.5)		
Poorly differentiated non-keratinizing carcinoma	5	4 (80)	1 (20)		
1-year distant metastasis				6.619	0.010
No	88	66 (75)	22 (25)		
Yes	8	2 (25)	6 (75)		
Locoregional recurrence					0.203
No	93	67 (72.0)	26 (28.0)		
Yes	3	1 (33.3)	2 (66.7)		
AJCC stage (2010)				3,079.5[a]	0.051
I	4	4 (100.0)	0 (0.0)		
II	7	5 (71.4)	2 (28.6)		
III	48	37 (77.1)	11 (22.9)		
IV	37	22 (59.5)	15 (40.5)		
T-stage				3,054.0[a]	0.032
1	13	12 (92.3)	1 (7.7)		
2	6	4 (66.7)	2 (33.3)		
3	49	36 (73.5)	13 (26.5)		
4	28	16 (57.1)	12 (42.9)		
N-stage				3,295.5[a]	0.983
0	7	5 (71.4)	2 (28.6)		
1	27	18 (66.7)	9 (33.3)		
2	46	35 (76.1)	11 (23.9)		
3	16	10 (62.5)	6 (37.5)		
M-stage				0.000	1.000
0	96	68 (70.8)	28 (29.2)		
1	0	0 (0.0)	0 (0.0)		
Chemotherapy[b]				0.024	0.878
No	23	16 (69.6)	7 (30.4)		
Yes	73	52 (71.2)	21 (28.8)		

[a]Wilcoxon W test. [b]With or without chemotherapy before/after radiotherapy. PD-L1, programmed death ligand 1; T, tumor; N, nodes; M, metastasis.

a particularly high incidence in southern China and Southeast Asia (11). After conventional treatment with chemoradiotherapy, the majority of NPC tumor cells are eliminated. The activation of immune cells, including tumor-infiltrating lymphocytes (TILs), is important for the elimination of residual tumor cells. However, a variety of immunosuppressive mechanisms act to reduce the activity of TILs in the tumor microenvironment, resulting in local recurrence and distant metastasis in a number of patients after radiotherapy and chemotherapy (12,13). Once this has occurred, effective treatment options are limited; the majority of patients in this situation choose palliative chemotherapy to prolong their survival period, but the effect is limited. Therefore, identifying how the immune suppression index is associated with the recurrence and metastasis of NPC may assist in improving the prognosis of patients.

Table II. Correlation of various markers in the serum with PD-L1 expression in tumor tissues.

A, EBV DNA levels in plasma vs. PD-L1 expression

Group	PD-L1 negative	PD-L1 positive	χ^2	P-value
EBV DNA$^-$	41	16	0.082	0.775
EBV DNA$^+$	27	12		

B, EBV antibody levels in serum vs. PD-L1 expression

Group	Cases (n)	Median	Mann-whitney U	P-value
EBV EA-IgA			762.5	0.152
PD-L1 negative	67	1.019		
PD-L1 positive	28	0.3405		
EBV VCA-IgA			694.0	0.046
PD-L1 negative	67	2.067		
PD-L1 positive	28	1.0155		

C, Lymphocyte subset levels in plasma vs. PD-L1 expression

Cell type	Cases (n)	Value (%)	t	P-value
CD3$^+$ T cells			0.38	0.705
PD-L1 negative	65	67.4754±9.608		
PD-L1 positive	26	66.6269±9.664		
CD3$^+$ CD4$^+$ T cells			0.39	0.697
PD-L1 negative	65	31.0446±7.672		
PD-L1 positive	26	30.3192±8.814		
CD3$^+$ CD8$^+$ T cells			0.09	0.929
PD-L1 negative	65	31.8554±9.731		
PD-L1 positive	26	31.6654±7.286		
CD4$^+$/CD8$^+$			768.0[a]	0.569
PD-L1 negative	64	0.965 (0.7575)		
PD-L1 positive	26	0.975 (0.52)		
NK cells			-0.741	0.460
PD-L1 negative	64	23.5734±10.0917		
PD-L1 positive	26	25.3731±11.2599		
CD3$^-$ CD19$^+$ cells			8.532[b]	0.014
PD-L1 negative	64	7.25 (5.95)		
PD-L1 low expression	19	8.1 (6.1)		
PD-L1 high expression	7	4.0 (2.9)		

Values are expressed as the mean ± standard deviation or median (interquartile range); [a]Mann-Whitney U test; [b]Kruskal-Wallis H test. Normal ranges: CD3$^+$T cells, 61.1-77.0%; CD3$^+$CD4$^+$ T cells, 25.8-41.6%; CD3$^+$CD8$^+$ T cells, 18.1-29.6%; CD4$^+$/CD8$^+$ cells, 0.98-1.94%; NK cells, 8.1-26.6%; CD3$^-$CD19$^+$ cells, 7.3-18.2%; EBV EA-IgA, <1.1 S/CO; EBV VCA-IgA, <1.1 S/CO. NK, natural killer; EBV, Epstein-Barr virus; PD-L1, programmed death-ligand 1; VCA-IgA, viral capsid antigen IgA; EA-IgA, early antigen IgA.

PD-L1 is a member of the Ig superfamily with the chromosomal location of 9p24.2, which encodes a 290-amino acid type I transmembrane protein, including an extracellular portion with IgV and IgC-like domains. Its IgV-like domain interacts with the extracellular IgV domain of PD-1, and the immune tyrosine motif region of the cytoplasmic tail of the PD-1 molecule serves an important role in the negative regulation of the immune response (14). Under normal physiological conditions, PD-1 maintains the body's immune tolerance as a negative regulator of T-cell proliferation; however, in tumors and viral infection, the overexpressed PD-L1 and PD-L2 on the cell surface interacts with PD-1 on

the surface of T cells to inhibit T-cell activation, proliferation and cytotoxicity to the tumor (15). A number of studies have demonstrated that the expression of PD-L1 is increased in numerous types of cancer, including breast cancer (16), renal cell carcinoma (17), ovarian cancer (18) and non-small cell lung cancer (19). The present study investigated the expression of PD-L1 in 96 NPC tumors and identified a PD-L1-positive rate of 29.2% (28/96). Previous Chinese and international studies regarding PD-L1 expression and clinical significance in NPC have reported conflicting results. Peng *et al* (20) identified a PD-L1-positive rate of 67.2% (43/64) in NPC tissue samples. This discrepancy with the results of the present study may be due to differences in the evaluation of PD-L1 staining, as in the present study, staining in <10% of the cells was considered to indicate a negative expression status. In addition, NPC has unique regional characteristics. The patients in the present study were from non-high incidence area (northern China) and there may be greater heterogeneity outside high-incidence areas.

To date, a range of studies have confirmed that PD-L1 expression is associated with the prognosis of cancer patients. Shi *et al* (21) identified that high expression of PD-L1 in colorectal carcinoma was associated with the tumor-nodes-metastasis stage and prognosis. Nomi *et al* (22) reported that pancreatic cancer patients with PD-L1-positive tumors exhibited a worse prognosis than those with PD-L1-negative tumors. Frigola *et al* (17) indicated that the expression of PD-L1 was associated with the tumor stage and prognosis in patients with renal cell carcinoma. Li *et al* (23) reported that high tumor expression of PD-L1 was associated with significantly poorer OS and DFS. Compared with other types of head and neck tumor, NPC has a higher risk of metastasis. Therefore, it is critically important to screen NPC patients who may have a high risk of recurrence and distant metastasis subsequent to conventional radiotherapy and chemotherapy. In the present study, it was identified that the expression of PD-L1 was not associated with sex, age, pathological type or lymph node metastasis, whereas it differed significantly depending on the T-stage of the primary tumor (P=0.032) and the 1-year distant metastasis status (P=0.01), suggesting that PD-L1-positive patients may have a relatively poor prognosis. The result that the association between the expression of PD-L1 and the clinical stage was not statistically significant (P=0.051) may be due to the small sample size. In addition, at the 1-year follow-up after radiotherapy, the survival analysis demonstrated that the DMFS for patients with tumors with a PD-L1-positive status was lower than that of the patients with tumors with a negative status (P=0.006).

The occurrence of NPC is highly associated with EBV infection; infection markers including EBV-DNA, as well as EBV EA-IgA and EBV VCA-IgA, have been widely used in the clinical diagnosis. In addition, the detection of T lymphocyte subsets in the peripheral blood of patients with malignant tumors by flow cytometry may be used to determine the immune status, which is a valuable reference for clinical diagnosis and treatment. The detection of EBV EA-IgA and EBV VCA-IgA antibodies is a common method for the clinical screening for and diagnosis of NPC. The presence of cell-free fragments of EBV-DNA in the serum has recently been identified to be valuable in predicting the prognosis and recurrence risk of NPC (24). In the present study, it was observed that the concentration of EBV VCA-IgA antibody in the serum of PD-L1-positive patients was significantly lower than that of PD-L1-negative patients (P=0.046), although the levels in each of the two groups were above the normal levels. In addition, the number of B lymphocytes ($CD3^-CD19^+$) in patients with PD-L1-positive status was significantly lower than that in patients with PD-L1-negative status. A possible explanation is that a PD-1/PD-L1 interaction mediated a negative regulation signal to produce immunosuppression, including the reduced secretion of the cytokines IL-2, IL-10 and IFN-γ (5), which may influence the number of serum B lymphocytes and the ability of B cells to produce antibodies subsequent to accepting a viral antigen. However, the specific mechanisms require further study. The present results suggest that the levels of EBV VCA-IgA and $CD3^-CD19^+$ cells may be used to predict PD-L1 expression, particularly in cases when PD-L1 cannot be detected due to a lack of a sufficient tissue sample.

In recent years, an increasing number of clinical trials on PD-L1 antibodies have been performed, and clinical remission has been achieved in certain patients with non-small cell lung cancer and renal cell carcinoma (25-27). However, no such trials have been performed on NPC, and targeted immunotherapy of the PD-1/PD-L1 pathway has not been fully evaluated in NPC. In the present study, it was observed that the positive expression rate of PD-L1 in NPC tissues was 29.2%, which was associated with distant metastasis and the T-stage of patients. The DMFS of patients with positive PD-L1 expression status was significantly reduced, suggesting a poor prognosis. Therefore, it may be hypothesized that blocking the PD-1/PD-L1 pathway may be effective as an immunotherapy for NPC. However, the present study was of a retrospective nature and had a small sample size, a short follow-up time and correspondingly a small number of recurrence and metastasis cases. In the future, a prospective study with a larger sample size, performed over a longer period, will be required, to allow for in-depth exploration of the association between PD-1/PD-L1 expression and groups of NPC patients with different prognoses, and to provide a theoretical basis for immunotherapy via the PD-1/PD-L1 pathway.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YQ and DW performed the experiments. YQ and YC analyzed and interpreted the patient data regarding NPC. LY and HL

performed the histological examination of NPC. DW and YC were major contributors in writing the manuscript. YC and WC conceived and designed the experiments. All authors read and approved the final manuscript.

Ethical approval and consent to participate

The present study was approved by the Institutional Review Board of the Cancer Hospital of the Chinese Academy of Medical Sciences (Beijing, China).

Consent for publication

Written informed consent was provided by all patients prior to enrollment in the study. The consent was provided by the guardians if they were under 18 years of age.

Competing interests

The authors declare no potential competing interests regarding this manuscript.

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