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Tumor microenvironment contributes to Epstein-Barr virus anti-nuclear antigen-1 antibody production in nasopharyngeal carcinoma

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Abstract. Nuclear antigen-1 (NA1) protein of Epstein-Barr virus (EBV) is expressed in EBV-infected cells in the microenvironment of cancer. Since immune cells infiltrate abundantly in nasopharyngeal carcinoma (NPC) tumor tissues, we hypothesized that the local tumor microenvironment may perform an important role in the production of antibodies directed at NA1. Furthermore, we hypothesized that anti-NA1 antibody originating in the local microenvironment could be secreted into the saliva of patients with NPC. In the present study, 20 healthy controls and 39 patients with NPC treated with intensity-modulated radiation therapy were recruited for the study. Saliva and serum samples were collected from the NPC patients, and nasopharyngeal tissue samples from the patients with NPC. The titers of anti-NA1 antibody [immunoglobulin A (IgA)] were determined by ELISA. Expression of NA1, human leukocyte antigen-antigen D related (HLA-DR), cluster of differentiation (CD)80, CD86, CD3, CD4, CD19 and IgA was detected by immunohistochemical staining on paraffin-embedded nasopharyngeal tissue sections. Anti-NA1 antibodies were detected in the serum and saliva samples of the patients with NPC. In infiltrating cells, expression of HLA-DR, CD80, CD86, CD3, CD4, CD19 and IgA was detected, indicating that dendritic cells, T lymphocytes and B lymphocytes were all present in the local tumor tissues. Furthermore, expression of EBNA1 protein was detected on the membrane of the NPC tumor cells. Therefore, the NPC tumor microenvironment has the potential to initiate a humoral response to EBNA1 by producing IgA antibodies.

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Introduction

Nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck malignant neoplasm that occurs globally, but is geographically distributed to a higher extent within South China and South East Asia. NPC is highly associated with Epstein-Barr virus (EBV) infection (1-5).

A number of different cell types can be infected by EBV, including B cells and epithelial cells (6,7). Following the natural infection with EBV, the virus executes a distinct program of gene expression in the lytic or latent cycles to establish a persistent infection. Nuclear antigen-1 (NA1) protein is expressed during latent and lytic cycles, binding to a replication origin within the viral genome. It also mediates replication and partitioning of the episome during the division of the host cell, and is essential for the maintenance of the viral genome in latency (8-11). Since NA1 protein is expressed in EBV-infected cells, including cancer cells or cells in the cancer microenvironment, it may act as an antigen to induce immune responses if the microenvironment is suitable for the induction of humoral responses directed at NA1 (7).

To elicit humoral immune responses to induce the production of NA1 antibodies in the tumor local environment, the elements essential for a humoral response should all be present. These elements include antigens (NA1 in this case), antigen-presenting cells (APCs), T helper cells and B cells (7). Since numerous immune cells infiltrate in the NPC tumor tissues, we hypothesized that the local tumor microenvironment may be adequate for the production of antibodies directed at NA1. Furthermore, since EBV is mainly transmitted via saliva, NA1 antibody originating in the local microenvironment may be secreted into the serum and saliva of patients with NPC (12). To confirm these hypotheses, serum samples and nasopharyngeal tissues were collected from patients with chronic inflammation with lymphoid hyperplasia and from patients with NPC, and antibodies directed at NA1 were detected. The results showed that NA1 antibodies were

detected in the serum and saliva samples of patients with NPC, and that the local production of the antibodies could be completed in part in the local tumor microenvironment.

Patients and methods

Patients. The research protocol was approved by the Institutional Review Board of West China University Hospital, Sichuan University (Chengdu, China). All patients provided written informed consent and agreed to be involved in the present study. Between September 2011 and April 2012, 39 patients (34 male, 5 female; mean age, 46.9; age range 22-69) with pathologically confirmed NPC from West China University Hospital were enrolled in the present study. Each study patient was evaluated by flexible fiberoptic endoscopic examination, magnetic resonance imaging scans of the head and neck, chest X-ray, bone scan and ultrasound of the abdomen prior to treatment. X-ray computed tomography scans of the chest/abdomen were performed when clinically indicated. The histology of the tumor was evaluated according to the World Health Organization classification (13). Tumors were staged by Tumor-Node-Metastasis classification and clinical staging according to the American Joint Committee on Cancer 2009 cancer staging classification (13). NPC patient characteristics are presented in Table I.

A total of 20 healthy individuals (15 females and 5 males) were recruited for the present study (mean age, 24.8 years; range, 20-34 years).

Sample collection. Blood and saliva samples were collected from the healthy volunteers and from the patients with NPC prior to treatment. Vein blood (1 ml) was collected into a sterile EDTA-containing vacutainer tube from each patient. Samples were then centrifuged at 400 x g and 4˚C for 10 min. The serum was collected, aliquoted and stored at -80˚C until use. At least 1 ml saliva was collected using a sterile container. Samples were then centrifuged at 400 x g and 4˚C for 10 min. The serum was collected, aliquoted and stored at -80˚C until use.

ELISA. Titers of antibodies directed at NA1 [immunoglobulin A (IgA)] in the saliva and serum samples were determined in duplicate by ELISA using commercial kits (anti-EBNA1 ELISA kit; catalog no. 03-01-60402) purchased from Zhongshan Bio-Tech Co., Ltd. (Guangzhou, China). Samples with antibody levels higher than the detection limits were diluted and measured again. In the Laboratory of Molecular and Translational Medicine, West China Second University Hospital, Sichuan University, Chengdu, China), the intra-assay and inter-assay coefficients of variation were ≤10% for all assays. The experiments were conducted by following the manufacturer's suggested procedures. Two wells of blank controls (sample dilution buffer), two negative controls and two positive controls (as provided in the kit) were included on each plate. Briefly, diluted saliva and serum samples were added to each well (100 μl in each well) of a 96-well plate. Following incubation at room temperature (RT) for 2 h on a microplate agitator rotating at 25 g, the plate was then washed with 1X PBS wash buffer with 0.05% Tween-20. Horseradish peroxidase (HRP)-conjugated anti-human IgA antibody from the kit (100 μl) was added to each well. Following incubation at RT for 20 min, the plate was then washed with 1X PBS wash buffer. Color development was conducted by addition of 100 μl tetramethylbenzidine substrate solution and incubation at RT for 10 min. Once the reaction was stopped, optical absorbance (OA) was immediately read on a spectrophotometer (Infinite M200; Tecan Trading AG, Männedorf, Switzerland) using 450 nm as the primary wavelength and 630 nm as the secondary wavelength. OA value at a wavelength of 450/630 nm for each test was determined.

Table I. Clinical characteristics of patients with NPC in the present study.

Characteristics	Patients with NPC
Total, n	39
Gender, n	
Male	34
Female	5
Mean age (range), years	46.9 (22-69)
Histology, n	
Squamous cell carcinoma, poorly differentiated, non-keratinized	38
Carcinoma, undifferentiated, non-keratinized	1
Tumor stage, n	
I	2
II	8
III	11
IV	18
Depth of tumor invasion, n	
T1	14
T2	6
T3	6
T4	13
N stage, n	
N0	2
N1	16
N2	14
N3	7
M stage, n	
M0	31
M1	8
Metastases, n	
Male	7
Female	1
Metastasis region, n	
Lung	2
Bone	4
Multiple regions (liver, lung, bone, lymph node)	2

NPC, nasopharyngeal carcinoma.

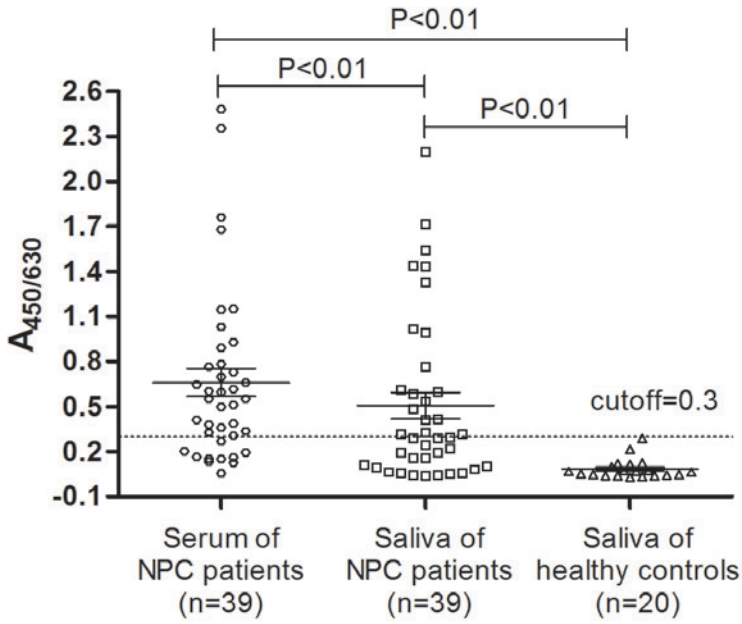

Figure 1. Optical absorbance value at wavelength 450/630 nm for anti-nuclear angiten-1 antibody in the saliva and serum samples of patients with NPC prior to anticancer treatment and in the saliva of healthy controls. NPC, nasopharyngeal carcinoma; A, absorbance.

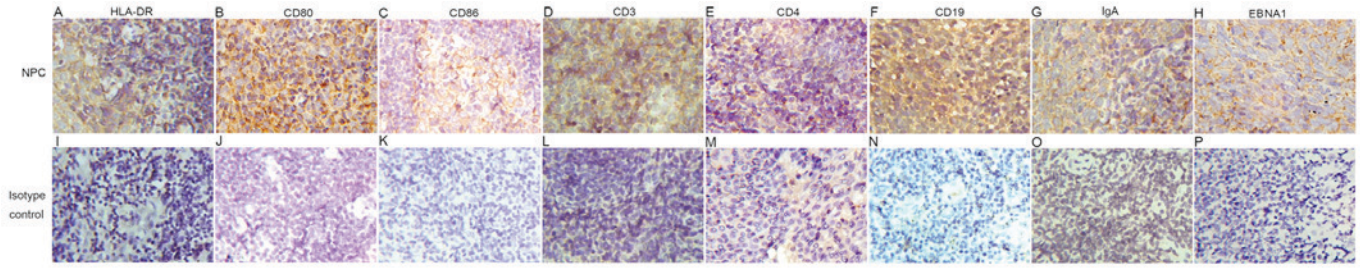

Figure 2. Expression of (A) HLA-DR, (B) CD80, (C) CD86, (D) CD3, (E) CD4, (F) CD19, (G) IgA and (H) EBNA1 was detected in the nasopharyngeal tissue by immunohistochemistry. When the primary antibody was replaced with the isotype control of (I) goat serum, (J) mouse IgG1, (K) rabbit IgG, (L) mouse (M) IgG2a or (N-P) κ, the positive signals were not observed (original magnification, x4,000). HLA-DR, human leukocyte antigen-antigen D related; CD, cluster of differentiation; IgA, immunoglobulin; EBNA1, Epstein-Barr virus nuclear antigen-1.

Immunohistochemistry. The immunohistochemical (IHC) staining was performed on paraffin-embedded nasopharyngeal tissue sections from 39 NPC patients. As shown in goat anti-human CD80 polyclonal antibody (1:100; IgG; catalog no. AF140; R&D Systems, Inc., Minneapolis, MN, USA), goat anti-human CD86 polyclonal antibody (1:100; IgG; catalog no. AF-141-NA; R&D Systems, Inc.), mouse anti-human CD4 monoclonal antibody (1:100; IgG1; catalog no. MAB379; R&D Systems, Inc.), rabbit anti-human CD3 polyclonal antibody (1:100; IgG; catalog no. ab5690; Abcam, Cambridge, UK), mouse anti-human CD19 monoclonal antibody (1:100; IgG2a, κ; catalog no. ab31947; Abcam), mouse anti-human human leukocyte antigen-antigen D related (1:100; HLA-DR) monoclonal antibody (IgG1; catalog no. ab20181; Abcam), mouse anti-human IgA monoclonal antibody (1:300; IgG1; catalog no. GTX17839; GeneTex, Inc., Irvine, CA, USA) or mouse anti-human EBNA1 antibody (1:300; IgG1; catalog no. GTX40777; GeneTex) were used as the primary antibodies for IHC staining. A negative control was set up using isotype control of goat serum (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), mouse IgG1, rabbit IgG, mouse IgG2a or mouse κ as the substitute for the primary antibody, respectively, according to the source of the primary antibody. All antibodies were diluted in PBS containing 0.1% bovine serum albumin (Roche Applied Science, Rotkreuz, Switzerland) and 0.01% sodium azide. Briefly, 4-μm tissue sections were deparaffinized for 15 min in xylene, hydrated in gradient ethyl alcohol of 100, 95, 85, 80 and 75%, and then incubated with 3% hydrogen peroxidase for 10 min at room temperature to block endogenous peroxidase. Following antigen retrieval in 0.1 mol/l citrate buffer (pH 6.0) for 15 min at 95˚C in a pressure cooker, sections were cooled to RT and washed three times with PBS, and then incubated with the aforementioned diluted primary antibodies at 4˚C overnight. Subsequently, sections were rewarmed to RT for 30 min and washed three times with PBS and then incubated with HRP-conjugated goat anti-rabbit/mouse secondary antibody (1:100; catalog no. ab6720; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 40 min at 37˚C. Diaminobenzidine was used at room temperature for 3 min for color development. Finally, the sections were counterstained with Mayer's hematoxylin and mounted in Permount (ZSGB-BIO, Beijing, China). Subsequently, light microscopy observation

(magnification, x4,000; 5 fields of view) was performed. The reactivity of cell type and location in the immunostained tissues were determined.

Statistical analysis. OA values at wavelength 450/630 nm of NA1 IgA in saliva and serum samples are expressed as the mean value ± standard deviation. The statistical significance of the findings was assessed by one-way analysis of variance using Prism 5 from GraphPad Software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Anti-NA1 in the serum and saliva. As shown in Fig. 1, antibody titers directed at NA1 were elevated in the saliva and serum of the majority of NPC patients, and anti-NA1 antibody titer in the serum was significantly higher than that of in the saliva of NPC patients ($P<0.01$). The cut-off value was defined as $A_{450/630}$ of 0.3. Compared with that of the saliva of the healthy control, the anti-NA1 antibody titer in the saliva of the NPC patients was significantly higher ($P<0.01$). Anti-NA1 antibody titer was determined to be negative in the saliva of the 20 healthy volunteers; however, 43.6% (17/39) of the patients with NPC were positive for the antibody. Furthermore, 74.4% (29/39) of the patients with NPC were positive for anti-NA1 antibodies in the sera.

Expression of HLA-DR, CD80, CD86, CD3, CD4, CD19, IgA, EBNA1 in the nasopharyngeal tissue. As shown in and Fig. 2, HLA-DR, CD80, CD86, CD3, CD4, CD19, IgA and EBNA1 antibodies were used as the primary antibodies for IHC staining. A negative control was set up using an isotype control of goat serum, mouse IgG1, rabbit IgG, mouse IgG2a or mouse κ as the substitute for each primary antibody, respectively, according to the source of the primary antibody.

When the control antibodies were used, no positive signals were noted on tissue sections from patients with NPC. In infiltrating cells, the expression of HLA-DR, CD80 and CD86 was detected at the membrane of dendritic cells, the expression of CD3 and CD4 was detected at the membrane of T lymphocytes, the expression of CD19, IgA was detected at the membrane of B lymphocytes and the expression of EBNA1 protein was highly expressed at the membrane of the tumor cells in NPC tissue specimens (Fig. 2).

Discussion

The level of serum antibodies directed at the EBNA1 antigen during latent and lytic cycles is elevated in patients with NPC and has been used as a serological marker for NPC diagnosis (14,15). Combined detection of anti-NA1, anti-early antigen (EA), anti-viral capsid antigen and anti-replication and transcription activator increases the sensitivity and has been considered as a useful serological method for NPC screening and diagnosis (16-20). Salivary IgA antibody titers were previously reported to be increased in response to EA, gp340 or NA1-p107 in healthy individuals compared with patients with NPC. However, these salivary parameters were poor in sensitivity and specificity for NPC screening and diagnosis (12,21,22). To the best of our knowledge, the present study is the first to report that anti-NA1 antibodies are elevated in the saliva and serum of patients with NPC. Although the detection of anti-NA1 antibody in saliva is non-invasive, quick and convenient, it has a limited value for clinical use due to its low sensitivity (12,21,22).

The present study investigated whether anti-NA1 antibodies could be induced in the local tumor environment. EBNA1 is the only viral protein consistently expressed during all forms of latency and in all EBV-associated malignancies (9,10). It is potentially a universal target for immune recognition of EBV-infected normal or malignant cells (10). The series of essential components for a humoral immune response, including NA1-expressed cells, APCs (HLA-DR+, CD80+ and CD86+), T helper cells (CD3+ and CD4+) and B cells (CD19+ and IgA+), were all detected by IHC staining in the local tumor environment. This indicated that anti-NA1 antibodies may be induced in the local tumor environment. The current study provides a novel insight into the use of EBNA1 antibodies and indicates that the tumor microenvironment contributes to the production of EBNA1 antibodies. Further study into the role of EBNA1 antibodies in the pathogenesis of EBV-related inflammation and NPC would, however, be useful to expand upon the present data.

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