• Clinical Research •

Epstein-Barr virus-encoded small RNAs in idiopathic orbital inflammatory pseudotumor tissues: a comparative case series

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Abstract

• AIM: To investigate the positive rate and types of cells that express Epstein-Barr virus-encoded small RNAs (EBERs) and to determine the distribution of EBER-expressing cells in idiopathic orbital inflammatory pseudotumor (IOIP) tissues.

• METHODS: We retrospectively examined 40 archived paraffin specimens from two teaching hospitals in Southern China between January 2007 and January 2015 that were pathologically determined to exhibit IOIP. Eleven concurrent paraffin specimens of thyroid-associated ophthalmopathy (TAO) composed the control group. *In situ* hybridization was performed to detect EBERs. Immunohistochemistry was employed to detect CD3, CD20, Vimentin, and smooth muscle actin (SMA), and the positive rate, types of positive cells, and distribution and location of EBERs were evaluated.

• RESULTS: The positive expression rate of EBERs was 47.5% (19/40) in the IOIP group, which was significantly higher than that in the TAO group [0 (0/11), *P*=0.011]. In the IOIP group, the lymphocyte infiltrative subtype, fibrotic subtype, and mixed subtype exhibited EBER-positive rates of 57.1% (12/21), 12.5% (1/8), and 54.5% (6/11), respectively, and no significant differences were found between these subtypes (*P*=0.085). Positive signals of EBERs were mainly present in medium-small lymphocytes between or around follicles and in the nuclei of activated immunoblasts (14/19).

• CONCLUSION: The positive rate, types, and distribution of EBER-expressing cells in IOIP have been documented. These findings are conducive for a better understanding of the underlying mechanisms of Epstein-Barr virus infection in IOIP pathogenesis.

• KEYWORDS: Epstein-Barr virus; Epstein-Barr virus-encoded small RNAs; thyroid eye disease; Graves' ophthalmopathy; idiopathic orbital inflammatory disease

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INTRODUCTION

Idiopathic orbital inflammatory pseudotumor (IOIP), also known as orbital pseudotumor, was first described by Birech-Hirsechfeld in 1905 as an intra-orbital inflammatory process that was non-specific and non-neoplastic[1-6]. Currently, the underlying pathological mechanisms of IOIP have yet to be fully understood[7-8]. Pender proposed that infection by Epstein-Barr virus (EBV) might be a trigger for multiple chronic autoimmune diseases[9-10]. Jin *et al*[11] found that the positive rate of EBV DNA in the plasma samples of IOIP patients was three times as much as control group. Nevertheless, the types of cells and their distribution and specific locations in IOIP tissues are not fully understood.

Epstein-Barr virus-encoded small RNAs (EBERs) are the most abundant viral transcripts in cells with latent EBV infection[12]. EBER detection using *in situ* hybridization is considered the gold standard for determining and locating latent EBV infection[12]. It has been demonstrated that persistent transcription of EBERs occurs in almost every EBV-associated lesion[13]. The goal of this study is to examine the EBER-positive rate, types of EBER-positive cells, and the distribution of EBER-positive cells in IOIP tissues.

SUBJECTS AND METHODS

Ethics Statement This retrospective study was approved by the institutional review board of the First Affiliated Hospital of Guangxi Medical University and followed the tenets of the Declaration of Helsinki. Written informed consent was provided by all study participants or their legal guardians.

Clinical Samples The archived paraffin specimens of 40 continuous IOIP cases, which were pathologically determined between January 2007 and January 2015 at the First Affiliated Hospital of Guangxi Medical University and Liuzhou People's Hospital, were used as the case group. In addition, the paraffin specimens of 11 patients with thyroid-associated ophthalmopathy (TAO) who underwent orbital decompression or strabismus surgery during the same period composed the control group. Pathological classification of IOIP was based on the method of Henderson[14], and inflammatory pseudotumor tissues were classified into three subtypes: lymphocyte infiltrative subtype, fibrotic subtype, and mixed subtype. The diagnostic criteria of TAO were based on those of Bartley and Gorman[15]. All specimens were fixed with 10% neutral formalin and then sliced into 4-μm paraffin-embedded sections. All tissue sections were subjected to hematoxylin-eosin and immunohistochemical staining for pathological confirmation. In addition, each paraffin-embedded specimen was used to generate five continuous sections, which were used for *in situ* hybridization of EBERs as well as for immunohistochemical staining of CD3, CD20, Vimentin, and SMA.

***In situ* Hybridization of Epstein-Barr virus-encoded small RNAs** REMBRANDT® *in situ* Hybridisation and Detection Kits (PanPath, Netherlands) were used to detect EBERs, and digoxin-labeled oligonucleotide probes were used to detect EBER1 and EBER2 according to the manufacturer's instructions.

Immunohistochemical Staining of CD3, CD20, Vimentin and SMA Monoclonal antibodies against CD3, CD20, SMA, and Vimentin and the GTVisionTM Secondary Antibody Kit (GeneTech, Shanghai, China) were used for immunohistochemical staining according to the manufacturers' instructions.

Statistical Analysis SPSS 13.0 software was used for statistical analyses. Chi-square tests were used to compare the count data between groups. Independent samples *t*-test were used to compare the age between groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

Epstein-Barr Virus-encoded Small RNA Expression in Idiopathic Orbital Inflammatory Pseudotumor and Thyroid-associated Ophthalmopathy The positive EBER expression of IOIP group was significantly higher than that of TAO group (47.5% *vs* 0, $P=0.011$) (Table 1). In IOIP group, the lymphocyte infiltrative subtype, fibrotic subtype, and mixed subtype had EBER-positive rates of 57.1% (12/21), 12.5% (1/8) and 54.5% (6/11), respectively, which were not significantly different ($P=0.085$).

Epstein-Barr Virus-encoded Small RNA-positive Cells in Idiopathic Orbital Inflammatory Pseudotumor *In situ* hybridization revealed that the EBER-positive cells in IOIP tissues could be divided into two subtypes. The first category was lymphocytes, which included small-medium-sized lymphocytes and activated immunoblasts. Observation of the continuous sections after Immunohistochemical staining revealed that some of these cells were $CD20^+$ B lymphocytes, although most of them could not be confirmed to express CD3 or CD20. The EBER-positive cells were mainly distributed in medium-small lymphocytes around follicles and in the nuclei of activated immunoblasts. In addition, three samples revealed that several ectopic folliculargerminal centers harbored 2-8 EBER-positive centroblasts. The second category included the spindle cells that proliferated in interstitial lesions and differentiated into fibroblasts/myofibroblasts. In total, three specimens with EBER-positive lymphocytes as well as EBER expression in the nuclei of mesenchymal spindle cells were observed (Figure 1). The density and distribution of EBER-positive cells in the 19 specimens are listed in Table 2. In contrast, *in situ* hybridization revealed that the inflammatory cells and spindle cells that infiltrated into extraocular muscle and caused hyperplasia were both EBER negative (Figure 2).

Table 1 The clinical features and EBERs expression in IOIP and TAO groups *n* (%)

Characteristics	IOIP group (*n*=40)	TAO group (*n*=11)	*P*
Gender			0.018[1]
Male	18 (45)	10 (90.9)	
Female	22 (55)	1 (9.1)	
Age (a)	49.1±20.2	39.3±13.8	0.136[2]
Unilateral or bilateral involvement			<0.001[1]
Unilateral	35 (87.5)	1 (9.1)	
Bilateral	5 (12.5)	10 (90.9)	
EBERs expression			0.011[1]
+	19 (47.5)	0	
-	21 (52.5)	11 (100)	

EBER: Epstein-Barr virus-encoded small RNA; IOIP: Idiopathic orbital inflammatory pseudotumor; TAO: Thyroid-associated ophthalmopathy. [1]Pearson's Chi-squared test with Yates' continuity correction; [2]Independent samples *t*-test.

DISCUSSION

Using *in situ* hybridization, we found that IOIP tissues exhibited a total EBER-positive rate of 47.5% (19/40), resulting from latent, high-frequency infection by EBV. The cell types of the EBER-positive population mainly included small-medium-sized lymphocytes and activated immunoblasts, although several specimens also exhibited ectopic folliculargerminal centers with EBER-positive centroblasts as well as spindle cells, which proliferated in interstitial lesions and differentiated into fibroblasts/myofibroblasts. The EBER-positive cells were mainly distributed between ectopic folliculargerminal centers and lymphocytes around follicles or disseminated in infiltrated lymphocytes. Because EBER-induced activation of the innate

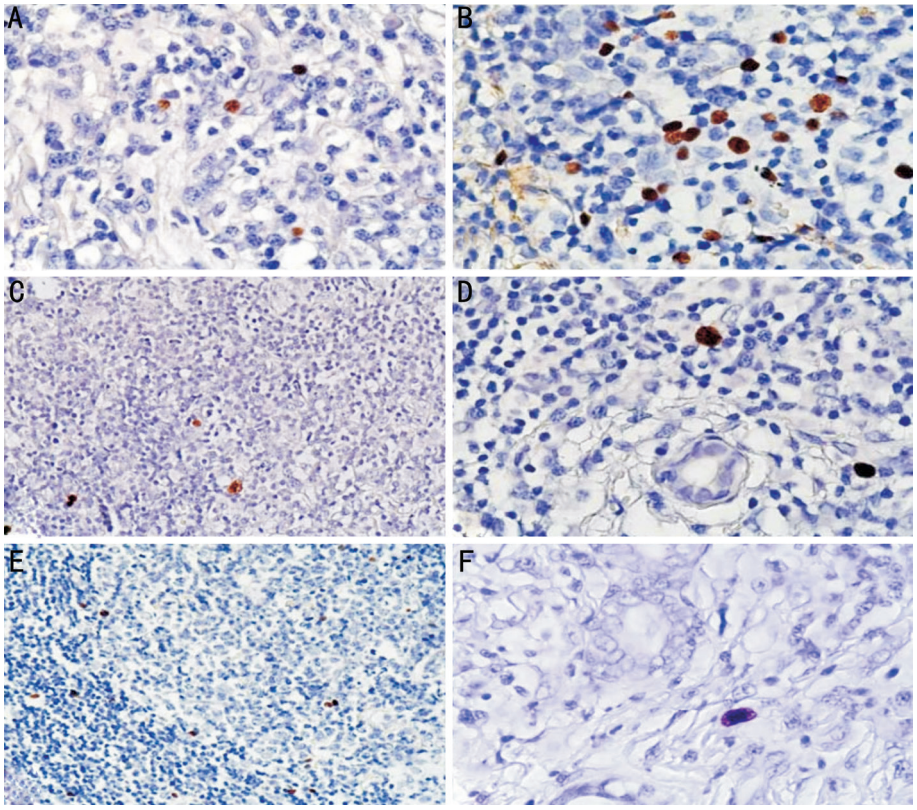

Figure 1 *In situ* hybridization revealed positive EBER expression in the following cells or locations A: Medium-small lymphocytes in IOIP tissues (×400); B: Medium-small lymphocytes in IOIP tissues as well as the nuclei of immunoblasts (×400); C: The nuclei of $CD20^+$ B immunoblasts (×200); D: The nuclei of activated immunoblasts (×400); E: Centroblasts in the germinal centers of ectopic lymphoid follicles as well as lymphocytes around the follicles (in nuclei) (×200); F: Nuclei of mesenchymal spindle cells and lymphocytes (×400).

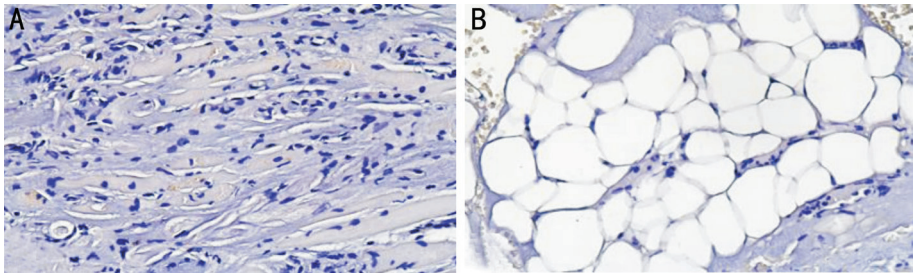

Figure 2 *In situ* hybridization revealed negative EBER expression in orbital tissues of the extraocular muscles of TAO patients A: Inflammatory cells and spindle cells (×200); B: Adipose hyperplasia (×200).

immune system may cause immunopathological diseases associated with EBV infection[13,16-17], our data further revealed that EBERs are closely related to IOIP pathogenesis.

In the lesions, ectopic lymphoid tissues emerged in the germinal centers of B-lymphatic follicles, and the follicles exhibited irregular enlargement. Among the 21 cases in which lymphocytes were the main EBER-positive population, 18 showed development of ectopic follicular germinal centers. In addition, in the 11 specimens with a mixed subtype, three manifested similar morphological changes. These findings indicated that autoreactive B cells exhibited specific clonal expansion in response to antigens in target organs. These lymphatic tissues may therefore become acquired lymphoid tissue under conditions of EBV infection and abnormal autoimmunity and continuously proliferate upon stimulation by antigens. The morphological changes in the IOIP lesions are consistent with the autoimmunity theory by Pender[18] regarding autoreactive B cells resulting from EBV infection. Specifically, the T cells resulting from local EBV infection repeatedly attack target organs, thereby generating ectopic lymphatic follicles with germinal centers in target organs. As a consequence, a large quantity of autoreactive B cells are produced, which may be the underlying cause of autoimmune diseases after EBV infection. Hence, Dreyfus[19] proposed a potentially effective anti-viral approach by employing virus-targeting drugs such as rituximab, which depletes memory B cells, or acyclovir, which is an anti-retroviral integrase inhibitor.

A previous study demonstrated that nine IOIP plasma specimens with different histological traits exhibited high levels of EBV DNA, although the data did not elucidate the types

Table 2 The density and distribution of EBER-positive cells in IOIP specimens

Case No.	Gender	Age (a)	Laterality	Pathological type	EBERs$^+$ cells/ section	Type and distribution of EBER$^+$ cells
1	Female	41	Right	Lymphocyte dominant	25	Inter-follicular small lymphocytes and immunoblasts
2	Male	39	Right	Lymphocyte dominant	80-100	Inter-follicular medium-small lymphocytes and immunoblasts
3	Male	32	Right	Lymphocyte dominant	5	Inter-follicular immunoblasts
4	Female	53	Right	Mixed	2	Inter-follicular immunoblasts
5	Male	35	Right	Lymphocyte dominant	>200	Inter-follicular small lymphocytes, medium-sized lymphocytes, and large immunoblasts, mesenchymal spindle cells, 8 positive centroblasts in 1 germinal center.
6	Female	39	Bilateral	Lymphocyte dominant	4	Inter-follicular small lymphocytes and immunoblasts
7	Female	70	Right	Lymphocyte dominant	7	Inter-follicular small lymphocytes and immunoblasts
8	Female	38	Bilateral	Lymphocyte dominant	2	Inter-follicular immunoblasts
9	Male	64	Bilateral	Lymphocyte dominant	>200	Inter-follicular small lymphocytes, immunoblasts, karyotypic irregular medium-sized lymphocytes and immunoblasts, mesenchymal spindle cells, 1-2 positive centroblasts in 2 germinal centers
10	Female	65	Left	Lymphocyte dominant	3	Inter-follicular small lymphocytes and immunoblasts
11	Female	60	Right	Lymphocyte dominant	2	Inter-follicular small lymphocytes and immunoblasts
12	Female	67	Right	Lymphocyte dominant	20	Inter-follicular small lymphocytes and immunoblasts
13	Male	35	Bilateral	Lymphocyte dominant	15	Inter-follicular small lymphocytes and immunoblasts
14	Female	68	Left	Mixed	12	Inter-follicular small lymphocytes and immunoblasts
15	Female	55	Left	Fibrous	5	Small lymphocytes
16	Male	69	Right	Mixed	11	Immunoblasts
17	Female	76	Left	Mixed	>200	Small lymphocytes, medium-sized lymphocytes, immunoblasts, and inside the nuclei of mesenchymal spindle cells
18	Female	84	Right	Mixed	40	Medium-small lymphocytes and immunoblasts
19	Male	56	Right	Mixed	6	Small lymphocytes

EBER: Epstein-Barr virus-encoded small RNA; IOIP: Idiopathic orbital inflammatory pseudotumor.

and distribution of EBV-infected EBER-positive cells[11]. Yan *et al*[20] used *in situ* hybridization to examine 37 IOIP specimens of the lymphatic infiltration subtype and found no hybridization signal using EBER mRNA. The author suggested that EBV possibly only played a role in the initiation of IOIP; once the tumor forms, the lymphocytes gradually lose genomic fragments or the entire genome of EBV, resulting the absence of EBERs in the lesion. Moreover, prolonged preservation of paraffin specimens may also cause loss of the EBER signal. Here, we used a standardized method for the preservation of paraffin specimens, and from morphological and molecular perspectives, we revealed that two major cell components of IOIP lesions, namely lymphocytes and spindle cells, both harbored EBERs, which are the transcripts of EBV (latent infection). This finding was complementary to previous IOIP pathological studies, which have not histologically shown the types and distributions of EBV-infected cells.

Due to limitations of the research conditions, we did not use double-labeling for *in situ* hybridization or immunochemistry to classify EBER-positive lymphocytes in IOIP lesions, although such a method might not necessarily facilitate complete differentiation between different types of EBER-positive cells. Niedobitek *et al*[17] reported that double-labeling of *in situ* hybridization and immunochemistry were used to detect non-neoplastic lymphoid tissues and revealed that most EBER-positive cells in the lesions could not be identified *via* B-cell- or T-cell-specific antibodies. Another limitation was that the number of male and females subjects in the TAO group was different because male subjects often had more severe ocular symptoms than females and required orbital decompression.

In summary, this study used histological and morphological approaches to demonstrate that high-level EBER expression was present in the IOIP lesions of humans. Furthermore, our data revealed the types, distributions, and locations of EBER-positive cells. These findings are conducive for a better

understanding of the underlying mechanisms of EBV infection in IOIP pathogenesis. Nonetheless, it remains to be determined whether the presence of EBERs indicates that IOIP patients are in the early stage of EBV reactivation.

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